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THE TOXIC EXTRACT OF THE MARINE FLAGELLATE *PRYMNESIUM PATELLIFERUM*

A Study of Its Effects on Cell Membrane Properties

MELDAHL Anne-Sophie

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THE TOXIC EXTRACT OF THE MARINE FLAGELLATE *PRYMNESIUM PATELLIFERUM*; A STUDY OF ITS EFFECTS ON CELL MEMBRANE PROPERTIES

by

ANNE-SOPHIE MELDAHL

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A new test method for the algal toxin desired from *Prymnesium* and *Chrysochromulina* has been developed. The toxin from *Prymnesium* interact with sodium and calcium channels in nerve ending particles. The toxic effect on fish could therefore be due to a disturbance of ion regulation of the fish gills.

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PREFACE

The work presented in this thesis was carried out at the Norwegian Defence Research Establishment (NDRE), Division for Environmental Toxicology in the period 1990-1994. The project entitled "Toxins of marine flagellates" was a part of the research program "Harmful Algae" initiated in 1988 by the Norwegian Council for Fisheries Research and finished in 1993.

I wish to express my gratitude to Professor Dr Philos Frode Fonnum, Head of the Division for Environmental Toxicology, NDRE, for excellent supervision, enthusiastic support, inspiration and constructive criticism during this work.

I would like to thank Dr Pål Aas for the interesting collaboration. Ms Rita Tansø and Ms Evy Grini Iversen are highly acknowledged for their expert technical assistance. I am also indebted to all my other colleagues at NDRE for support, co-operation and friendliness.

I am indebted to Professor Eystein Paasche and Professor Jahn Throndsen, Heads of the Department of Marine Botany, University of Oslo, for providing research facilities. I sincerely thank Dr Bente Edvardsen for the interesting collaboration and for all the helpful discussions regarding the ecology and biology of the marine flagellates. My thanks are also extended to the entire staff at the Department of Marine Botany for support and friendliness.

I am grateful to Professor Olav Sand, Jørgen Kolderup and Stig Eriksen at the Department of Physiology, University of Oslo, and Professor Egil Haug and Ms Nina Gjerlausen at the Hormone Laboratory, Aker Hospital, for the valuable contributions to this project.

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Anne-Sophie Meldahl
October 1995

CONTENTS

THE TOXIC EXTRACT OF THE MARINE FLAGELLATE *PRYMNESIUM PATELLIFERUM*; A STUDY OF ITS EFFECTS ON CELL MEMBRANE PROPERTIES

1 OBJECT OF THE INVESTIGATION

Harmful algal blooms have been observed with increasing frequency in Norwegian coastal waters during the last years. In 1988 an large toxic bloom of *Chrysochromulina polylepis* in the Skagerrak/Kattegat area caused extensive damage both to fish aquaculture and to native marine flora and fauna. In 1989 a toxic bloom of *Prymnesium patelliferum* and *P. parvum* threatened for the first time the aquaculture industry in the fjords of Ryfylke, south-western Norway and 750 tons of salmon and rainbow trout were killed. Blooms of these and related algal species toxic to fish have since then caused the death of large amounts of reared salmon and rainbow trout. At the beginning of the present study little was known about the factors that were important for the formation of such algal blooms, the production of their toxins and the mechanisms underlying the biological effects of the toxins. Previous studies of the toxicity of *P. parvum* had indicated that the cell membranes of the fish gills were the primary targets of the algal toxin. The aims of the present study were to develop a sensitive method for detection of the toxins of such ichthyotoxic algae and further to study their toxic effects on biological cell membranes. The present investigations were carried out using *P. patelliferum* as a model for the closely related potentially toxic algal flagellates. The potent inhibitory effect of the toxic extract of this alga on the transport of the neurotransmitters L-glutamate and GABA in a preparation of rat brain nerve endings (synaptosomes) formed the basis of the development of a sensitive method for the detection of ichthyotoxins (Papers I and II). Further, using this and other nerve-cell preparations, the effects of the toxic extract of *P. patelliferum* on various cell membrane properties were investigated (Papers III, IV and V) in order to approach an understanding of the mechanism underlying the toxic effect on the fish.

2 LIST OF PAPERS

This thesis is based on the following papers which will be referred to in the text by their Roman numerals:

(I) Meldahl, A.-S. and Fonnum, F. (1993) Effect of toxin of *Prymnesium patelliferum* on neurotransmitter transport mechanisms. Development of a sensitive test method. *J. Toxicol. Environ. Health,* **38,** 57-67.

(II) Meldahl, A.-S., Edvardsen, B. and Fonnum, F. (1994) Toxicity of four potentially ichthyotoxic marine phytoflagellates determined by four different test methods. *J. Toxicol. Environ. Health* **42**, 289-301.

(III) Meldahl, A.-S. and Fonnum, F. The effects of a purified toxic extract of *Prymnesium patelliferum* on transport of ions through the plasma membrane of synaptosomes. *Toxicon* **33**, 1071-1086.

(IV) Kolderup, J., Meldahl, A.-S., Eriksen, S., Haug, E., Fonnum, F. and Sand, O. Toxin of the marine alga *Prymnesium patelliferum* enhances voltage dependent Ca^{2+}-currents, elevates the cytosolic Ca^{2+}-concentration and facilitates hormone release in clonal rat pituitary cells. *Acta Physiol. Scand.* **154**, 321-327.

(V) Meldahl, A.-S., Aas, P. and Fonnum, F. Extract of the marine alga *Prymnesium patelliferum* induces release of acetylcholine from cholinergic nerves. *Eur. J. Pharm.* (submitted).

3 GENERAL INTRODUCTION

3.1 Blooms of toxic algae

Of the thousands of living phytoplankton species that make up the base of the marine food chain, only about 40 have the capacity to produce potent toxins. Most are dinoflagellates, prymnesiophytes, diatoms or chloromonads. Blooms of toxic algal species can cause harm to humans, via clams, mussels, oysters or scallops and in certain cases fish which ingest the algae as food and retain the toxins in their tissues. Typically the shellfish and the fish themselves are only marginally affected, but a single clam can sometimes accumulate enough toxin to kill the human who eats it. There are several different shellfish poisoning syndromes, and they have been described as paralytic, diarrhetic, neurotoxic and amnesic shellfish poisoning, abbreviated PSP, DSP, NSP and ASP, respectively (for review see Hallegraeff, 1993).

Phytoplankton can also kill marine animals directly, and those that have the potential to produce toxins which are lethal to fish are termed **ichthyotoxic** phytoplankton. Farmed fish are especially vulnerable because the caged animals cannot avoid the blooms. In Norway, blooms of the species *Prymnesium parvum, Prymnesium patelliferum, Chrysochromulina polylepis and Chrysochromulina leadbeateri* have been responsible for the death of hundreds of tons of farmed fish since 1988 (Eikrem and Throndsen, 1993), and have caused large economic losses for the fish-farming industry. While the toxicity of *P. parvum* has been known since 1938 (Otterstrøm and Steenman-Nielsen, 1939), both species of

Chrysochromulina were previously unknown as toxin producers. The *Prymnesium* species *P. patelliferum* and *P. parvum* have appeared together in toxic blooms in the brackish water of the inner fjords of Ryfylke, south-western Norway, during July and August each year since 1989. It is mainly the farmed salmon and rainbow trout that have been affected during these blooms of *Prymnesium* (Kaartvedt *et al.*, 1991; Aksnes, 1993) and during the bloom of *C. leadbeateri* in Lofoten in May 1991 (Johannessen *et al.*, 1991; Tangen, 1991). In contrast, during the toxic bloom of *C. polylepis* in Kattegat and Skagerrak in May-June 1988 a wide range of marine organisms as well as salmonid fish in fish farms were killed (Dahl *et al.*, 1989; Underdahl *et al.*, 1989).

There is a conviction among many experts that the scale and complexity of toxic algal blooms is expanding. The number of toxic blooms, the economic losses and the kinds of toxins and toxic species have all increased (Smayda and Wyatt, 1995). Blooms of harmful algae is not a new phenomenon; until 1970, some 1600 cases of human paralytic shellfish poisoning had been recorded world-wide, mostly in North America and Europe (Prakash *et al.*, 1971). Since then more than 900 additional cases have been reported, many occurring in regions of the world where paralytic shellfish poisoning had not been known (WHO, 1984; Hallegraeff, 1993). The reason for the apparent increased frequency of toxic algal blooms in the last twenty years, and whether human activities have contributed, is still a highly debated question. However, there is today no doubt that our increasing interest in utilising coastal waters for aquaculture is leading to an increased awareness of toxic algal species.

With respect to the *C. polylepis* bloom in 1988, it is becoming increasingly likely that the toxic bloom was due to exceptional climatic and hydrographic conditions at that time (e.g. bright and warm weather, strong stratification with brackish water in the upper layer, elevated nitrogen levels and high nitrogen to phosphorus ratio in the upper layer) (Dahl *et al.*, 1989; Granéli *et al.*, 1993). *Chrysochromulina polylepis* was present in the Skagerrak area also in 1989, 1990, 1991, 1992 and 1994 (Fyllingen *et al.*, 1993; Granéli *et al.*, 1993; HOV, 1994) but there was no sign of another serious toxic bloom of this species. This indicates that a long term disturbance of the environment due to the trend of increasing nutrient concentration in this area in the years preceding the 1988 bloom (Anderson and Rydberg, 1988; Barth and Nielsen, 1989), is probably not of major importance for the development of such toxic blooms. Moreover, the toxic bloom in 1991 of another *Chrysochromulina, C. leadbeateri*, developed in the unpolluted waters of northern Norway, supporting the assumption that large scale eutrophication is not a prime requisite for harmful blooms of these flagellates.

It has been suggested that the development of the ichthyotoxic blooms of *Prymnesium* species in the fjord system of Ryfylke, south-western Norway, may have been stimulated by nutrient load, especially phosphate, from the fish farms (Kaartvedt *et al.*, 1991). The spreading of the algae through the fjord system was then driven by freshwater released from a hydroelectric power plant. The freshwater supplying the brackish fjord had high

concentrations of silicate and nitrate but low concentrations of phosphate. Hence, it was suggested that a high ratio of nitrogen to phosphorus and phosphorus deficiency was important for the production of toxin by *Prymnesium* (Kaartvedt *et al.*, 1991; Aure and Rey, 1992), as had previously been demonstrated for laboratory cultures of *Prymnesium* (Shilo, 1967).

3.2 *Prymnesium patelliferum* or *Prymnesium parvum* ?

Prymnesium patelliferum, P. parvum, C. polylepis and C. leadbeateri belong to the kingdom Protista, division Haptophyta, class Prymnesiophyceae, subclass Prymnesiophycidae, order Prymnesiales and family Prymnesiaceae (for references see Jordan and Green, 1994)

Initially the ichthyotoxic algal blooms in the fjord system of Ryfylke were thought to be due to *P. parvum*, and in most reports the blooms are still ascribed to this species. Transmission electron microscopy examinations of scales from cells collected in the area, revealed, however, the additional presence of *P. patelliferum* (Eikrem and Throndsen, 1993). The morphological difference between *P. parvum* and *P. patelliferum* lies in the surface pattern of the scales that covers the algae (Green et al., 1982). Otherwise they are practically identical. Despite the fact that both species were present in the water samples collected during the blooms, the establishment of axenic cultures by the dilution technique (Throndsen, 1978) at the Division for Marine Botany, University of Oslo, repeatedly yielded only *P. patelliferum* and not *P. parvum*. This strain of *P. patelliferum* has been used in all of the present investigations (Papers I-V), and the strain of *P. parvum* used in the work of Paper II originated from a bloom in Fladesø, Denmark. Recently, however, cultures of *P. parvum* originating from the Ryfylke bloom material were established by the capillary isolation technique (A. Larsen, University of Bergen).

The toxicity of *P. parvum* has been amply documented (for review see Paster, 1973; Shilo, 1982), whereas virtually no information has been published on the toxicity of *P. patelliferum*. Since the two species can not be separated by light microscopy, the traditional method for species identification, it is quite likely that some of the earlier reports on *P. parvum* in reality were dealing with *P. patelliferum*. Therefore, in the following the term *Prymnesium* will be used to indicate either or both species. Figure 3.1 shows schematic drawings of *Prymnesium* as well as the two *Chrysochromulina* species *C. polylepis* and *C. leadbeateri*.

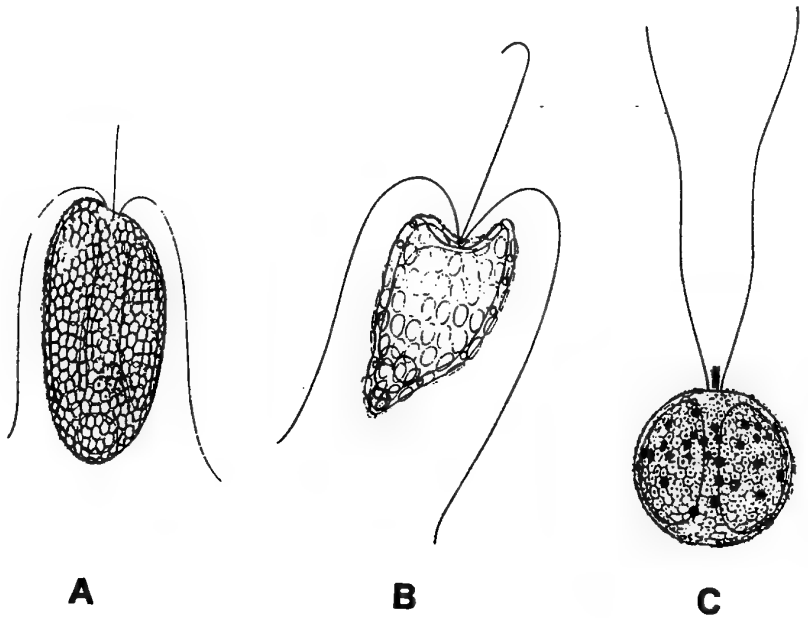


Figure 3.1 Drawings of (A) Prymnesium (P. parvum and P. patelliferum), (B) Chrysochromulina polylepis, (C) Chrysochromulina leadbeateri, showing cell shape and scale cover. The drawings were kindly provided by Dr Jahn Throndsen, Division for Marine Botany, Department of Biology, University of Oslo.

3.3 The ichthyotoxicity of *Prymnesium*

In the early literature describing the various toxic effects of *Prymnesium* the terms *Prymnesium*-toxin or prymnesin were used to indicate a toxic extract of the algae. The expression *Prymnesium*-toxin will be used in this thesis when referring to the studies were this term has been used. It should, however, be kept in mind that the early experiments were carried out using organic extracts of different purity and some of the toxic extracts used may have contained more than one toxic entity (see also section 3.5). The term *Prymnesium*-toxin will be used for simplicity also when referring to the toxic extracts of *P. patelliferum* used in the present studies.

The ichthyotoxicity of *Prymnesium* was first documented by Otterstrøm and Steenmann-Nielsen (1939) who showed that the toxic effect of the algae was caused by a compound that was excreted to the water. Since then, cases of fish mortality due to blooms of *Prymnesium* in brackish water have been reported from many areas of the world such as Israel (Reich and Aschner, 1947), Bulgaria (Valkanov, 1964; Petrova, 1965), England (Farrow, 1969; Holdway et al., 1978), the former Soviet Union (Krasnoshchek and Abramovich, 1971), Germany (Hickel, 1976; Dietrich and Hesse, 1990), Spain (Comin and Ferrer, 1978), Denmark (Bach and Jacobsen, 1991), Finland (Lindholm and Virtanen,

1992), and several times from Norway (reviewed by Aksnes, 1993; Eikrem and Throndsen, 1993).

During the early historic development of a sensitive assay for the determination of the ichthyotoxicity of *Prymnesium*, it was shown that the expression of ichthyotoxic activity was dependent on the presence of a cofactor such as the divalent cations Ca^{2+}, Mg^{2+} or streptomycin (Yariv and Hestrin, 1961). The authors did not explain the reason why these compounds were examined and the mechanism underlying their potentiating effect on ichthyotoxicity of *P. parvum* remains obscure. Ulitzur and Shilo (1964) extended the list of activating cofactors of ichthyotoxic activity to include the cationic polyamines such as spermine and other related polyamines. However, these compounds have also been reported to act as membrane stabilising agents in a variety of biological systems such as bacterial spheroplasts and protoplasts, mitochondria and microsomes (Tabor et al., 1961), spermine being most active in all these systems. In agreement with the latter report Shilo (1971) found that spermine indeed protected protoplasts of *Mycoplasma* from lysis by *Prymnesium* toxin. In recent years numerous reports have addressed the influence of polyamines on several different membrane functions (reviewed by Schuber, 1989).

The earliest recognisable effect of *Prymnesium* ichthyotoxin on the intact fish was found to be loss of the selective permeability of the gill tissue, allowing entrance of trypan blue and macromolecules such as [^{131}I]human serum albumin into the gill tissue (Ulitzur and Shilo, 1966). Trypan blue uptake in the *Prymnesium* treated fish was found to be dependent on the presence of the different cationic substances such as spermine, 3,3-diaminopropylamine or $MgCl_2$. The requirement for cofactors appeared necessary only for the effect of the toxin on immersed fish, since the toxicity by intraperitoneal injection of the toxin into fish showed no such cofactor requirements (Bergmann et al., 1963; Ulitzur and Shilo, 1966). The increased permeability of the fish gills could be observed within minutes (less than 5 minutes) (Ulitzur and Shilo, 1966). The damage of the gill permeability was found to be reversible; pre-treated fish transferred into toxin-free conditions for 4 to 8 hours gradually lost the enhanced gill permeability. It was thus concluded that the intoxication of gill-breathing animals immersed in water containing the *Prymnesium*-toxin consist of two stages; (i) an initial reversible damage to the gill tissue resulting in the loss of their selective permeability and (ii) a second stage, leading to death due to increased sensitivity to a number of toxicants that may be present in the milieu, including the *Prymnesium*-toxin. The initial step was shown to occur only in the presence of an activating factor such as spermine or related polyamines (Ulitzur and Shilo, 1966). The property of the fish to recover from sublethal doses of the *Prymnesium*-toxin has been used also by the fish-farmers in the Ryfylke area during toxic blooms of *Prymnesium* in recent years, by moving the fish-pens from the affected area to a *Prymnesium*-free area.

3.4 Effects and stability of *Prymnesium*-toxin *in vitro*

Several different effects of the toxic extract of *Prymnesium* have been shown in vitro. The effects depend on the particular cell-system to be studied. The toxic action appeared to be directed towards biological membranes, and in animal cells its final expression when present in high concentrations over extended periods of time was lysis of the cell. In contrast, intact bacteria were resistant to lysis, probably due to the protection by their cell-wall (Shilo, 1971). *Prymnesium* toxin was shown to elicit lytic effects on erythrocytes (Yariv and Hestrin, 1961) and a number of nucleated cells, including Ehrlich ascites cells, HeLa cells, normal human liver cells, and amnion cells (Shilo and Rosenberger, 1960; Dafni, 1971; Aune, 1989). Treated Ehrlich ascites cells swelled, formed pseudopodia-like extrusions, stained with trypan blue and finally lysed (Shilo and Rosenberger, 1960). It was suggested that the swelling and early damage to Ehrlich ascites tumour cells in the presence of *Prymnesium* toxin was due to increased permeability to ions such as K^+ and Na+ and an increase in the total intracellular content of these ions (Dafni and Giberman, 1972). Further, using artificial bilayer membranes, Moran and Iliani (1974) found that the toxin caused a marked increase in membrane conductance, and the membrane became permeable to cations. The hypothesis was put forward that aggregates of the toxin were intercalated into the membrane to form negatively charged aqueous pores.

Neurotoxic effects of the toxin of *Prymnesium* have been demonstrated using different preparations of nerves and muscles such as the smooth muscle of guinea-pig ileum (Bergmann et al., 1964), the frog sartorius muscle with its sciatic nerve (Parnas and Abbot, 1965), and the deep extensor abdominal medialis muscle of the crayfish and the lobster (Parnas and Abbot, 1965). In all these systems the toxin seemed to act on the postsynaptic membrane of the end plate which lead to a decline in the mechanical response of the muscle to indirect stimulation. In the isolated guinea-pig ileum there was, however, a dual effect consisting of an initial enhancement of the contraction of the muscle which lasted 4-5 minutes, followed by inhibition of the contraction in response to acetylcholine (Bergmann et al., 1964). The question was therefore raised whether the multiplicity of toxic manifestations observed for *Prymnesium*-toxin in diverse biological systems was due to different expressions of one compound, or a mixture of different toxic compounds.

Detailed investigation of the heterogenity of the toxin extracted from *Prymnesium* was carried out by Reich et al. (1965). It was possible to selectively abolish the inhibitory effect on the acetylcholine-induced contraction of the guinea-pig ileum described above, before dramatically affecting the hemolytic or the ichthyotoxic activity by subjecting the toxin to a variety of inactivation procedures such as irradiation by visible and ultraviolet light, incubation at 37 °C and exposure to alkaline media. The curve describing this inactivation of the inhibitory activity of ileum contractions was different in shape depending on whether the toxin was deactivated with visible light, UV light, 37 °C or high pH, and it was suggested that different parts of the active molecules were affected depending on the deactivation procedure. The photoinactivation of the ichthyotoxic activity of the

Prymnesium-toxin had previously been found to occur both in an atmosphere of pure nitrogen or pure oxygen (Parnas et al., 1962), indicating that oxidation of the toxic compounds was probably not involved. *Prymnesium*-toxin was shown to be more stable when dissolved in organic solvents than in aqueous solution (Shilo, 1971), and both the ichthyotoxic and the hemolytic activity of *Prymnesium* were protected from inactivation by visible light in the presence of methanol (>10%).

In our laboratory we have recently studied the stability of the toxic extract of *P. patelliferum*, and the results obtained were similar to the results described above. The toxic extract was exposed to different inactivation procedures, and the toxic activity of the algal extract was then determined by its inhibitory effect on the uptake of the neurotransmitter L-glutamate into synaptosomes of rat brain. The toxic extract of *P. patelliferum* was deactivated by incubation at pH 4 or pH 10 as well as by the treatment with visible light or UV light (Meldahl et al., 1995). The deactivation occurring during the 24 hour exposure to visible light was fully prevented by the presence of 50% methanol, but methanol had no protective effect during the three hour exposure to UV irradiation. Therefore, during the course of the investigations presented in this thesis, the toxic extract of *P. patelliferum*, and also those of the related algae used in Paper II, were stored in 100% methanol and protected from exposure to light during incubation in water solutions. When stored in methanol the toxic activity remained unchanged at temperatures up to 90 °C (Meldahl, unpublished observations). Separation of the toxic extract of *P. patelliferum* on thin layer chromatography plates revealed the presence of 6 hemolytic spots (Meldahl, unpublished observations), which had previously bee shown also for the *Prymnesium*-toxin studied by Ulitzur and Shilo (1970). Our results thus indicated that the crude extract of *P. patelliferum* contained toxic compounds similar to the *Prymnesium* used in the previous studies in the laboratories of Parnas and Shilo.

3.5 Formation and isolation of toxins of *Prymnesium*

As shown by Reich and Aschner (1947) there is no simple relationship between the *Prymnesium* count and the toxicity of the water. A high number of algal cells does not always mean high toxicity of the surrounding water body, or vice versa. When grown in a suitable medium, synthesis of the toxic principles is most pronounced during the late stages of the logarithmic phase of growth and continues into the stationary phase (Shilo, 1967). It was thus reasoned that the toxin production could be related to limitation of growth. Indeed, limitation of phosphorus enhanced toxin formation even before markedly affecting growth (Shilo, 1967). A ten- to twenty-fold increase in toxin (determined as hemolytic, cytotoxic and ichthyotoxic activity) was found in phosphate starved cells, although total cell yields were lower than in phosphate-rich media. Limiting levels of nitrogen, thiamine or vitamin B12 in the media did not cause any increase of the toxin production (Shilo, 1971). In the study of Igarashi et al. (1995), it was shown, however, that with strong

aeration of the algal culture (air flow of 100 ml/s) both cell density and toxin yield per cell was improved nearly 20 fold.

The toxic syndromes caused by seafood poisoning due to toxic marine phytoplankton are most often due to families of compounds of various degree of similarity present in the alga. For instance, at least 18 different saxitoxins which cause paralytic shellfish poisoning (PSP) with widely different potencies are produced by some species of the dinoflagellate *Alexandrium* (Yang and Kao, 1992). Also in *Alexandrium* the composition and the amounts of toxin vary with the growth conditions (Anderson, 1990).

Several classes of toxins have been isolated from *Prymnesium* (Table 3.1). Using a standard procedure for polar-lipid purification the toxic principle was purified 1000-3000-fold with respect to specific hemolytic, cytotoxic and ichthyotoxic activity, with a yield of 50% of initial activity (Ulitzur and Shilo, 1970). The chemical properties of the isolated compound were found to be similar to those of acidic polar lipids, and the toxin was suggested to be a proteolipid. Chemical analysis of the toxin revealed 15 amino acids, a number of unidentified fatty acids, 0.47% phosphate and 10-12% hexose sugars (Ulitzur and Shilo, 1970). Separation of this toxic extract by thin layer chromatography showed six spots with hemolytic activity when the plate was sprayed with a suspension of red blood cells. Three of these spots contained protein and all contained phosphorus (Ulitzur and Shilo, 1970). Using a slightly different method for isolation, but also based on differential extraction with organic solvents, Paster (1968) isolated a toxic principle which gave only a single peak in analytical ultracentrifugation. It had only 1/20 the specific hemolytic activity of the toxin prepared by Ulitzur and Shilo (1970). The chemical analysis of this compound showed 70% sugar hexoses, especially glucose and galactose, and 30% fatty acids, but no phosphorus, nitrogen, or sulphur. The compound described by Paster (1968) may possibly have been an additional toxic factor produced by *Prymnesium*.

More recent studies using modern chromatographic techniques have resulted in the isolation of the hemolytic compounds giving rise to the strongest hemolytic spot on the thin layer chromatography plate (hemolysin 1) (Kozakai et al., 1982). Hemolysin 1 was described as a mixture of two galactoglycerolipids named hemolysin 1a and 1b (Figure 3.2A). The two hemolysins, varying only in the degree of saturation of the attached fatty acid chain, were closely related to those isolated from the ichthyotoxic algae *Chrysochromulina polylepis* and Gyrodinium aureolum (Yasumoto et al., 1990). In a recent preliminary work, Igarashi et al. (1995) isolated two other toxins from *Prymnesium* with polyene-polyether-structure, named prymnesin-1 and prymnesin-2 (Figure 3.2B). The two latter toxins were over 1000 times more hemolytically active than saponin, lethal to both fish and mice and induced Ca^{2+}-influx into C6 rat glioma cells. No information was given in this preliminary report on whether prymnesin-1 and prymnesin-2 constitute one or two of the six hemolytic entities of the crude organic extract. Although only partly described, the chemical structures of prymnesin-1 and -2 have much in common with the structure of many of the polyether dinoflagellate toxins such as the diarrhetic shellfish toxins (Lee et

al., 1989), the brevetoxins (Baden, 1989), and ciguatoxin (Legrand, 1991). Moreover, similarly to the *Prymnesium* toxins, both brevetoxins and ciguatoxin are potent ichthyotoxins (Shimizu et al., 1986; Lewis, 1992).

Chemical class of toxin	Molecular weight	Biological activities	Content	References
Lipopolysaccharide (glycolipid with polysaccharide moiety)	23 000 ± 1800*	Ichthyotoxic Hemolytic Lethal to mice (i.p.)	70% sugar hexoses (glucose, galactose), 30% fatty acids, no P, N or S	Paster (1968)
Acidic proteolipid		Ichthyotoxic Hemolytic Cytotoxic	10-12% sugar hexoses, unidentified fatty acids, 15 amino acids, 0.47 % P_i	Ulitzur and Shilo (1970)
Glycolipids: hemolysin 1a hemolysin 1b	 675 (1a) 673 (1b)	Hemolytic		Kozakai *et al.* (1982)
Polyether-polyenes: prymnesin-1 prymnesin-2	 2264 (prym-1) 1970 (prym-2)	Ichthyotoxic Hemolytic Lethal to mice (i.p.) Induce influx of Ca^{2+} into C6 rat glioma cells		Igarashi *et al.* (1995)

Table 3.1 Some properties of toxic compounds isolated from Prymnesium
**Calculated from sedimentary velocity*

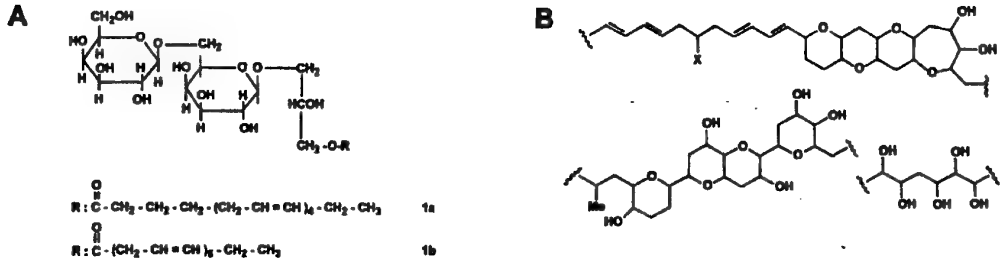


Figure 3.2 Structures of biologically active compounds isolated from Prymnesium. (A) Structure of hemolysin 1a and 1b (Kozakai et al., 1982). (B) Probable partial structures of N-acetylprymnesin-2. X: O or N (Igarashi et al., 1995).

3.6 Detection of ichthyotoxins

The general effect on the cell membrane of the toxin of *Prymnesium* have permitted the use of many different cell types for the detection of the toxin. Consequently, there is a wide variation in the values reported for the effective concentrations of algal toxin. This variation may also be due to differences in algal growth conditions, and procedures for extraction of the toxic activity.

An assay system for detection of the ichthyotoxicity of *Prymnesium* based on fish and tadpoles placed into a solution of ichthyotoxin was first established (Shilo and Aschner, 1953). The finding that certain polycationic substances were able to enhance the ichthyotoxicity of the *Prymnesium* toxin (see section 3.3.) allowed the detection of sublethal quantities of ichthyotoxin (Yariv and Hestrin, 1961). To avoid the interference of cofactors, and to reduce the amount of toxin required, Bergmann et al. (1963) developed a method for direct intraperitoneal injection of the toxic extract into fish. This method proved to be more accurate and reproducible. Recently, a method using larvae of the crustacean Artemia salina was found to be a simple and sensitive method for the detection of toxicity of cultures of both *Chrysochromulina* and *Prymnesium* (Edvardsen, 1993; Larsen et al., 1993; Paper II).

The method for determination of hemolytic activity was for a long time considered the most convenient method for assaying the toxin of *Prymnesium*. Erythrocytes of many vertebrates as well as from fish have been used. The method is rapid and can be performed both quantitatively, by measuring the hemoglobin in the supernatant at 540 nm in a spectrophotometer, and qualitatively by visual observation of the colour change of the supernatant. Moreover, the principle of hemolytic activity has been useful for detection of hemolytic activity on paper chromatograms and thin layer chromatography plates. When

thin-layer chromatographic plates are sprayed with a suspension of erythrocytes, hemolytic areas of the algal toxin show up as white spots on a pale red background (Shilo, 1971).

A test using hepatocytes from rats has been used for the detection of toxic activity in extract of *C. polylepis*, *C. leadbeateri* and *P. parvum* (Underdahl et al., 1989; Aune et al., 1992). The extracts of the algae were incubated with the freshly prepared hepatocytes at 37 °C for 2 hours. The toxicity was determined by morphological studies of the cell membranes and by measuring the leakage of the cytosolic enzyme lactate dehydrogenase. The toxic effects of the extracts of both algae (membrane disruption and staining with trypan blue) were easily separated from the effects of the well known diarrhetic shellfish poison (DSP) complex (blebbing of the membrane and no staining with trypan blue) (Aune, personal communication). Relatively high concentrations of toxin of *Prymnesium* and *Chrysochromulina* are required to detect these effects which are determined after two hours.

In the present thesis a test method using a nerve-cell preparation of rat brain (synaptosomes) has been employed (Papers I, II and III). The extracts of the algae were incubated with freshly prepared synaptosomes at 30 °C for 15 min. The toxicity was determined as inhibition of the uptake of the neurotransmitter substances L-glutamate or γ-aminobutyric acid (GABA) into the synaptosomes. Much lower amounts of algal toxins can be detected with this method compared to the test methods based on the lysis of erythrocytes (Paper I) or rat hepatocytes (Underdahl et al., 1989).

4 MAIN FINDINGS IN THIS THESIS

Paper I:

The toxin of *P. patelliferum* was shown to inhibit the uptake of neurotransmitters into synaptosomes and synaptic vesicles isolated from rat brain. The two test methods were about 100 and 10 times more sensitive, respectively, than the method based on determination of hemolysis of human red blood cells. All three test methods showed that toxin production in *P. patelliferum* was increased by growth under phosphorus limitation.

Paper II:

Four different test methods have been compared for the determination of the toxicity of the related potentially ichthyotoxic algae *P. patelliferum*, *P. parvum*, *C. polylepis* and *C. leadbeateri*. *Chrysochromulina* leadbeateri in culture was non-toxic and differed from *C. leadbeateri* in the natural ichthyotoxic outbreak. The growing toxic algal cultures were lethal to larvae of A. salina, and the toxic algal extracts were hemolytic and inhibited neurotransmitter transport in synaptosomes and synaptic vesicles. The results indicated that similar toxins are produced by *P. patelliferum*, *P. parvum* and *C. polylepis*. None of the test

methods responded to *C. leadbeateri*, indicating that the test systems are not affected by general metabolites of the algal flagellates.

Paper III:

The extract of *P. patelliferum* was purified on an aminopropyl silica column. The extract inhibited the uptake of L-glutamate into synaptosomes, increased the permeability to Na^+, K^+ and Ca^{2+} and caused membrane depolarisation of synaptosomes. The concentration of algal toxin causing 50 % effect in the various assays increased in the following order: Na^+-influx < L-glutamate uptake < plasma membrane depolarisation ≈ Ca^{2+}-influx < K^+-efflux. The Na^+-influx, the Ca^{2+}-influx and the membrane depolarisation induced by the algal extract were inhibited by pre-treatment of the synaptosomes with the Na+/Ca^{2+}-channel blocker flunarizine. There was no significant effect of the algal extract on the Na^+/K^+-ATPase of the synaptosomes.

Paper IV:

Using the whole-cell voltage clamp technique it was shown that the toxic extract of *P. patelliferum* increased the voltage dependent Ca^{2+}-current in clonal anterior pituitary cells (GH4C1). In parallel to this effect there was an increase in the intracellular concentration of Ca^{2+} and secretion of prolactin. All three effects were inhibited by the Ca^{2+}-channel blockers D-600 (100 μM) or verapamil (100 μM) which suggests that voltage-activated Ca^{2+}-channels were activated by the algal toxin.

Paper V:

The toxin of *P. patelliferum* enhanced both the basal efflux and the K^+-evoked release of acetylcholine from the cholinergic nerves in a preparation of smooth muscle of rat bronchi. The induction of acetylcholine release under resting conditions was Ca^{2+}-independent and was potentiated by the lipophilic agents flunarizine, procaine and sphingosine. The enhancement of the K^+-evoked release was suppressed by removal of Ca^{2+} as well as by the Ca^{2+}-channel blockers ω-conotoxin, diltiazem, nifedipine and flunarizine. The results indicate that a part of the acetylcholine-release induced by the algal toxin may be due to an activation of voltage-activated Ca^{2+}-channels.

5 GENERAL DISCUSSION

In the present study it has been shown that toxin present in lipid extracts of the ichthyotoxic algal flagellates *P. patelliferum*, *P. parvum* and *C. polylepis* exhibit similar effects on cell membranes. In addition to the hemolytic activity they inhibit the transport of the neurotransmitters L-glutamate and γ-aminobutyric acid (GABA) into synaptosomes as well as the transport of L-glutamate, GABA and dopamine into synaptic vesicles (Papers I and II). The mechanisms of action underlying these effects have been studied in more detail using the toxic extract of *P. patelliferum* as a model. The effects of this algal extract

on transport mechanisms over the cell plasma membrane have been investigated using synaptosomes of rat brain (Paper III) as well as two other preparations; clonal cells of the rat anterior pituitary (Paper IV) and pieces of smooth muscle of the rat bronchus (Paper V). On the basis of the present investigations, this discussion will focus on the following points: The mechanism behind the observed alteration of the neurotransmitter transport mechanisms with emphasis on the effect on ion permeability (5.1), the possible effect of the toxic extract on voltage-gated Ca^{2+}-channels (5.2 and 5.3), and on protein phosphorylation (5.4), the cytolytic (5.5) and the ichthyotoxic effect (5.6) and finally, the use of synaptosomes in a test method for toxins of the ichthyotoxic marine flagellates (5.7).

5.1 The inhibition of neurotransmitter uptake by *Prymnesium*-toxin

The overall process of synaptic transmission may be divided into three stages: (a) release of transmitter into the synaptic cleft, (b) its interaction with post-synaptic receptors, and (c) removal of the transmitter from the synaptic cleft. The neurotransmitter substances L-glutamate and GABA can be removed from the synaptic cleft by reuptake into the nerve ending. The uptake of the neurotransmitters L-glutamate and GABA into the nerve endings have been studied in detail (Kanner and Shuldiner, 1987). L-Glutamate and GABA are taken up by distinct carrier proteins (transporters) (Guastella et al., 1990; Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992). With respect to the driving forces for the transport mechanism, they are similar for GABA and L-glutamate in that both are coupled to the transmembrane Na^+-gradient maintained by a Na^+/K^+-ATPase, and both are dependent on the membrane potential (Figure 5.1). However, while an outward directed K+-gradient is required for the net uptake of L-glutamate, the net uptake of GABA is independent on K^+ but dependent on external Cl-. The transporters show high affinity for their substrates (Km in the low μM range).

Inside the nerve terminal neurotransmitters are taken up and stored in a concentrated form in synaptic vesicles (see Figure 5.1). The vesicles accumulate L-glutamate, GABA and dopamine by means of transporter molecules that differ from those of the plasma membrane. The vesicular uptakes are driven by a Mg^{2+}-ATP dependent process which generates an electrochemical proton gradient (Toll and Howard, 1978; Naito and Ueda, 1983, 1985; Fykse and Fonnum, 1988). In contrast to the uptake of neurotransmitters over the plasma membrane of synaptosomes, the neurotransmitter uptake systems of synaptic vesicles are independent of sodium and potassium and show low affinity for their substrates (Km in the low mM range).

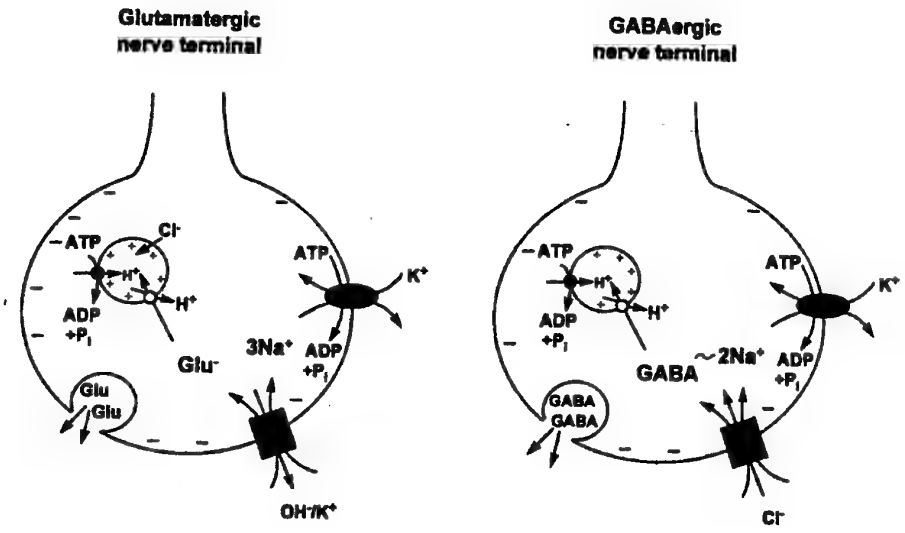


Figure 5.1 Schematic model of the transport of the amino acid neurotransmitter substances L-glutamate and GABA into the nerve terminal and the intracellular synaptic vesicles

The extract of *P. patelliferum* proved to be a potent inhibitor of five different transport systems for neurotransmitters: the uptake of L-glutamate and GABA over the synaptosomal plasma membrane, and the uptake of L-glutamate, GABA and dopamine into synaptic vesicles (Paper I). Two main hypotheses could be put forward for the mechanism underlying these effects: First, the algal toxin may have acted by direct binding to the transporter molecules. This possibility seems unlikely since all the transporter molecules are different. The second possibility, which appears more likely, would be that the algal toxin is able to disturb one or several of the membrane properties that the various transport processes are dependent on to different degrees. The effects of the algal toxin on different membrane properties of synaptosomes such as the permeability to Na^+, Ca^{2+}, K^+ and cytosolic lactate dehydrogenase (LDH), the membrane potential and the Na^+/K^+-ATPase were therefore investigated (Paper III). In contrast to the release of neurotransmitter from the nerve terminal, the process of neurotransmitter uptake over the nerve cell plasma membrane is independent of Ca^{2+}. The effects of the algal extract on the Ca^{2+}-permeability will therefore be discussed in a separate section (5.2).

5.1.1 The effects of *Prymnesium*-toxin on membrane properties of synaptosomes

The importance of the electrochemical gradient of Na^+ as a driving force for the high affinity uptake system for neurotransmitters is well established (Kanner and Sharon, 1978; Brew and Attwell, 1987; Kanner and Shuldiner, 1987; Nicholls and Attwell, 1990). Hence, the transport is sensitive to ionophores such as monensin, gramicidin D and nigericin which collapse the Na^+-gradient (Kanner and Sharon, 1978; Roskoski et al., 1981) and to ouabain which inhibits the Na^+/K^+-ATPase (Stallcup et al., 1979). Moreover, the

neurotoxin veratridine which opens the voltage dependent Na^+-channel, inhibits transport of L-glutamate and GABA. Tetrodotoxin, which blocks the Na^+-channel, reverses this inhibition (Kanner, 1980). Transport is enhanced by valinomycin which hyperpolarizes the membrane (Roskoski et al., 1981).

The toxin of *P. patelliferum* caused a persistent reduction of the inward Na^+-gradient, the outward K^+-gradient and the membrane potential of the synaptosomes (Paper III). These effects were not due to inhibition of the Na^+/K^+-ATPase or leakage through large holes in the membrane, since there was no significant effect of the algal extract neither on the ion-pumping enzyme or on the permeability to cytosolic LDH (Paper III). Each of the three effects (increase in Na^+-influx, K^+-efflux and membrane depolarisation) would contribute to net efflux of neurotransmitter from the cytoplasm by reversal of the plasma membrane transporters (Nicholls and Attwell, 1990), which in the present investigations was observed as inhibition of neurotransmitter uptake. It is difficult to determine which is the primary effect of the algal toxin on the synaptosomal membrane and which may be secondary effects. The algal toxin, however, was shown to be similarly effective in reducing the synaptosomal Na^+-concentration gradient as in inhibiting the uptake of L-glutamate into the synaptosomes, whereas the IC50 for effects on the K^+-gradient and the membrane potential were significantly higher (Paper III). Hence, the reduction in the Na^+-gradient is probably mainly responsible for the inhibition of the uptake of both L-glutamate and GABA into synaptosomes. It may be suggested that also the membrane depolarisation occurred as a consequence of the increase in Na^+-influx. Further, it is possible that the observed increase in K^+-efflux and Ca^{2+}-influx in the presence of the algal extract may have occurred through voltage-sensitive K^+- and Ca^{2+}-channels, respectively, which opened as a response to the membrane depolarisation. Thus, from the results of the work presented in Paper III the following sequence of effects of the algal extract on the synaptosomes could be suggested:

P. patelliferum extract → Na^+-influx ↑
- ↱ inhibition of neurotransmitter uptake
- ↳ membrane depolarisation
  - ↱ Ca^{2+}-influx ↑
  - ↳ K^+-efflux ↑

The involvement of voltage dependent Ca^{2+}-channels is supported by several findings discussed in section 5.2. The increase in Na^+-influx by the algal extract was insensitive to the Na^+-channel blocker tetrodotoxin (Paper III), indicating that voltage-sensitive Na^+-channels were not affected. Whether the blockers of the voltage-sensitive K^+-channels such as 4-aminopyridine and tetraethylammonium chloride would inhibit the K^+-efflux mediated by the algal extract remains to be investigated.

5.1.2 The effects of *Prymnesium*-toxin on membrane properties of synaptic vesicles

Small synaptic vesicles contain carrier systems capable of taking up their specific neurotransmitter against a concentration gradient. Common to the vesicular transport of neurotransmitters is the requirement of an electrochemical proton gradient, i.e. inside positive (for review see Njus et al., 1986; Maycox et al., 1990), generated by an Mg^{2+}-activated H^+-ATPase of the vacuolar type present in the vesicle membrane (Cidon and Shira, 1989; Floor et al., 1990). The accumulation of neurotransmitters occurs via four classes of neurotransmitter transporters: one specific for the biogenic amines, one for acetylcholine, one for L-glutamate and one for GABA and glycine (Fonnum et al., 1992). Uptake of the negatively charged L-glutamate is driven largely by the membrane potential difference and is stimulated by low concentrations of Cl- (Fykse et al., 1989; Tabb et al., 1992). Uptake of the positively charged dopamine is driven largely by the pH gradient, and uptake of GABA requires both components of the electrochemical proton gradient, i.e. the membrane potential and the pH gradient (Hell et al., 1990).

It was shown that the extract of *P. patelliferum* inhibited the vesicular uptake of L-glutamate, GABA and dopamine (Paper I). Due to the high uptake efficiency for dopamine, this uptake was measured using about 2-4 times less vesicles than used for the measurements of L-glutamate and GABA uptake (Paper I). Under comparable experimental conditions, however, it was found that the inhibition of dopamine uptake by the algal extract was similar to that of L-glutamate uptake (Meldahl, unpublished observations). The vesicular uptake of GABA was significantly less affected (Papers I and II). Thus, the sensitivity of the three different uptake systems to inhibition by the toxic extract followed the order: dopamine uptake $\approx$ L-glutamate uptake > GABA uptake.

It was therefore of interest to investigate the effects of the toxic extract on membrane properties that are known to be important for the vesicular uptake process, such as the pH gradient, the membrane potential and the proton pumping Mg^{2+}-ATPase, and to see whether one or more of these parameters would be especially sensitive to the toxic extract. In preliminary experiments it was found that the toxin of *P. patelliferum* at a concentration of 50 000 cells/ml caused 86% reduction of the vesicular pH gradient and 42% membrane depolarisation (Meldahl, unpublished observations). No effect on the proton pumping Mg^{2+}-ATPase was detected at this concentration of algal extract. Hence, it may be suggested that the toxin of *P. patelliferum* acts on the synaptic vesicles similarly to a protonophore which would be expected to depolarise the vesicles due to the H^+-efflux (Cidon and Shira, 1989). This explanation does, however, not explain why the uptake system for GABA was significantly less affected by the algal extract than were the uptake systems for L-glutamate and dopamine, since the uptake of GABA is known to be dependent on both the pH gradient and the membrane potential, whereas the L-glutamate uptake is driven mainly by the membrane potential and the dopamine uptake mainly by the pH gradient.

A clear interpretation of the effects of the toxic extract of *P. patelliferum* on the neurotransmitter uptake into synaptic vesicles will have to await a more complete understanding of the mechanisms for the different uptake systems. For example, the exact mechanism by which low concentrations of Cl- stimulate the vesicular uptake of L-glutamate is still unknown. This property of the uptake of L-glutamate may be important for understanding the effect of the algal extract on this uptake system.

The toxic extracts of *P. parvum* and *C. polylepis* showed similar differential inhibitory activity on the vesicular uptake of L-glutamate and GABA (Paper II) indicating that this is a property which is common to toxins produced by these related marine algae.

5.2 The effect of *Prymnesium*-toxin on voltage-dependent Ca^{2+}-channels

Calcium plays an important role as an intracellular messenger to trigger the release of neurotransmitters (Augustine et al., 1987) and neurohormones (Douglas, 1968). Four main types of voltage activated Ca^{2+}-channels of the nerve cell membrane have been characterised on the basis of pharmacological and electrophysiological investigations, named L-, N-, P- and T-type Ca^{2+}-channels (see Fox et al., 1987; Tsien et al., 1988; Llinás et al., 1989, Mintz et al., 1992). The L-type channel is blocked by the dihydropyridines (nifedipine), benzothiazepines (diltiazem) and phenylalkyl amines (verapamil, D600). The N-channel, which is believed to be important for the triggering of neurotransmitter release, is insensitive to the organic Ca^{2+}-blockers, but is blocked by the peptide ω-conotoxin GVIA. The P-type channel is blocked by the peptide ω-agatoxin. There is no selective blocker for the T-type channel, although in some preparations blockade of the T-type channels by the diphenylalkylamine flunarizine has been demonstrated (see section 5.3).

There were several results in the present study which indicated that the extract of *P. patelliferum* may increase the influx of Ca^{2+} into the cell through the voltage-dependent Ca^{2+}-channels (Papers III, IV and V). The most convincing results were obtained using the patch-clamp technique on clonal rat anterior pituitary cells (GH_4C_1-cells) in culture (Paper IV). Addition of the algal extract caused an increase in voltage-dependent Ca^{2+}-currents through the T-and L-type channels as well as an increase in intracellular Ca^{2+}-concentration as measured by fura-2 microfluorometry. Further, the algal toxin induced release of prolactin, in accordance with the well recognised relationship between elevation of $[Ca^{2+}]_i$ and hormone secretion in pituitary cells (see Ozawa and Sand, 1986). These effects on the GH_4C_1-cells were all inhibited by 100 μM of the L-type Ca^{2+}-channel blockers D-600 or verapamil (Paper IV).

The effect of the algal toxin on Ca^{2+} homeostasis was also investigated in two other systems. First, in Paper V it is shown that the enhancement of the K^+-induced release of [3H]acetylcholine from the rat bronchial cholinergic nerves induced by the algal extract was dependent on extracellular Ca^{2+} and was suppressed by blockers of the voltage-dependent Ca^{2+}-channels such as ω-conotoxin GVIA, nifedipine, diltiazem and flunarizine.

Thus, the toxin apparently provoked influx of extracellular Ca^{2+} in this preparation. Second, in rat brain synaptosomes the increase in intracellular Ca^{2+}-concentration by addition of the algal extract was dependent on extracellular Ca^{2+} (Paper III). This effect was blocked by the Na^{+}/Ca^{2+}-channel blocker flunarizine, but no inhibition was observed by other Ca^{2+}-channel blockers such as verapamil, nifedipine, diltiazem or ω-conotoxin. The latter results were similar to the small effect of verapamil, nifedipine and diltiazem on depolarisation-induced Ca^{2+}-influx in synaptosomes found by others (Nachshen and Blaustein, 1979; Daniell et al., 1983; Miller and Freedman, 1984; Rampe et al., 1984; Carvalho et al., 1988). This result did therefore not permit a detailed investigation of the effect on voltage dependent Ca^{2+}-channels in synaptosomes.

Altogether, the results of these investigations indicate that *P. patelliferum* produces toxin(s) which may open the voltage-activated Ca^{2+}-channels of the nerve cell membrane. It is interesting to note that the toxin of the closely related species *P. parvum* induced Ca^{2+}-influx into rat glioma C6 cells (Igarashi et al., 1995). Although no further details were given in this communication regarding the specific target of the algal toxin, the similar effect of toxins of *P. parvum* and *P. patelliferum* on membrane-flux of Ca^{2+} supports the assumption of a close relationship between the toxins of these morphologically related species (Paper II).

In should be noted, however, that the concentrations of the Ca^{2+}-channel blockers used in the present investigations were probably too high to be selective for the various types of Ca^{2+}-channels. Thus, no conclusions can be drawn regarding the types of Ca^{2+}-channels that may be affected by the extract of *P. patelliferum*. The electrophysiological measurements in pituitary cells showed, however, an increase in current by addition of the toxic extract which could be ascribed to activation of both the T- and the L-type of Ca^{2+}-channels, suggesting that the algal toxin probably interacts with structures common for the activation of both these channel types (Paper IV).

5.3 The dual effect of flunarizine

Flunarizine (1-[bis(p-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl)piperazine) is a highly lipophilic basic diphenylalkylamine belonging to class III of the Ca^{2+}-channel blockers (Spedding, 1985). It is widely acknowledged as a Ca^{2+}-channel blocker in a number of tissues (reviewed by Todd and Benfield, 1989). This compound appeared to inhibit the increase in the intracellular $[Na^{+}]$ and $[Ca^{2+}]$ as well as the membrane depolarisation of synaptosomes exerted by the extract of *P. patelliferum* (Paper III). Moreover, in another investigation (Paper V), flunarizine effectively inhibited the Ca^{2+}-dependent potentiation by the algal extract of the K^{+}-induced release of [^{3}H]ACh from the smooth muscle preparation. These results are consistent with the previously reported inhibition by flunarizine of K^{+}-induced increase in $[Ca^{2+}]i$ (Wibo et al., 1983; Carvalho et al., 1989; Kobayashi et al., 1992; Cousin et al., 1993) as well as inhibition of veratridine- and

ouabain-induced Na^+-influx (Cousin et al., 1993). The mechanism of action of flunarizine remains speculative. Although shown to be a specific blocker of the transient T-type Ca^{2+}-channel in preparations such as N1E-115 mouse neuroblastoma cells (Wang et al., 1990) and isolated hippocampal neurones (Takahashi and Akaike, 1991), flunarizine is considered non-specific as Ca^{2+}-channel antagonist. Our finding that flunarizine reduced the alteration in Na^+ and Ca^{2+} permeability as well as depolarisation in synaptosomes induced by the algal extract supports this notion. The increase in synaptosomal permeability to K^+ in the presence of the algal extract, measured as increase in $^{86}Rb^+$ efflux, was however, insensitive to inhibition by flunarizine (Paper III) suggesting that flunarizine is not likely to act as a general ion channel protector.

To our surprise, the efflux of [^{3}H]acetylcholine from cholinergic nerves in the smooth muscle preparation induced by the algal extract under resting conditions, was strongly potentiated in the presence of both 10 and 100 μM flunarizine (Paper V). This result is difficult to understand, but as discussed in Paper V it may be due to a lipophilic interaction between flunarizine and the algal toxin which may facilitate the interaction of the algal toxin with the cell membranes. Moreover, two other lipophilic compounds, procaine (Na^+-channel blocker) and sphingosine (inhibitor of protein kinase C), which were examined because of their potential in modulating acetylcholine release during depolarisation, had similar potentiating effect when present together with the algal extract under normal physiological conditions. It is, therefore, possible that also these synergistic effects were due to a lipophilic interaction between the algal toxin and procaine or sphingosine which facilitated the effect of the toxin on the cholinergic nerves.

5.4 The effect of *Prymnesium*-toxin on the phosphorylation of synaptosomal proteins

Protein phosphorylation and dephosphorylation represent fundamental and ubiquitous regulatory mechanisms of cellular activity. The process of synaptic transmission involves several steps and diverse molecules which could be modulated by phosphorylation. There is now substantial evidence to support a regulatory role for protein phosphorylation/ dephosphorylation in neurotransmitter release (Llinás et al., 1985; Dunkley and Robinson, 1986; Hemmings et al., 1989; Dekker et al., 1990; Robinson, 1991; Sihra et al., 1992; Hens et al., 1993). Despite this association, the precise roles of synaptosomal protein kinases, protein phosphatases and their substrates have not been determined.

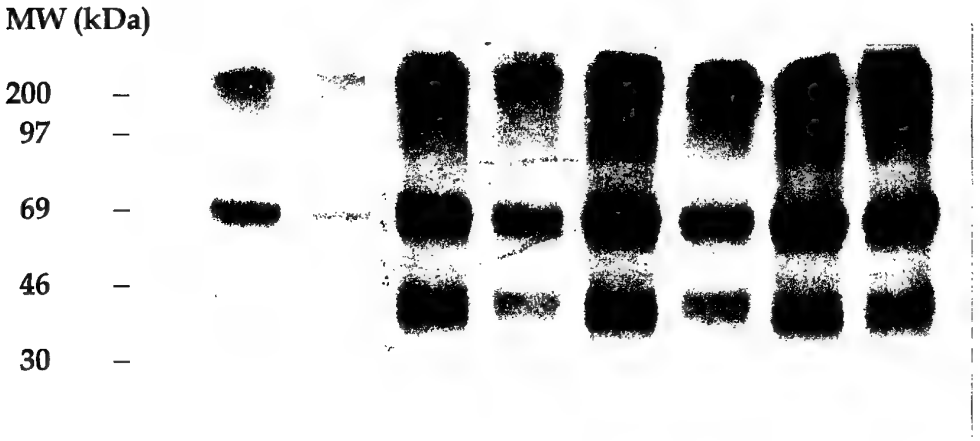
Okadaic acid, a dinoflagellate-toxin (Yasumoto et al., 1984), has been a useful tool in exploring the role of phosphorylation and dephosphorylation of proteins in synaptic transmission. It is a potent inhibitor of two of the four main protein phosphatases, protein serine/threonine phosphatases 1 and 2A (reviewed in Cohen et al., 1990). It is interesting to

note that many of the effects of the extract of *P. patelliferum* found in the present investigations show similarities with effects of okadaic acid. Analogous to the facilitation of ACh-release induced by the extract of *P. patelliferum* in the bronchial smooth muscle preparation (Paper V), it has been shown that okadaic acid enhanced neurotransmitter release at frog (cholinergic) and lobster (glutamatergic and GABAergic) neuromuscular junctions (Abdul-Ghani et al., 1991) and induced release of amino acid neurotransmitters from rat brain synaptosomes (Sim et al., 1993). Moreover, the observed enhancement of Ca^{2+}-currents in the clonal pituitary cells by the toxic extract of *P. patelliferum* (Paper IV) is similar to the marked increase in the whole-cell Ca^{2+}-current in myocytes from guinea-pig heart by okadaic acid (Hescheler et al., 1988). Elevation of $[Ca^{2+}]i$ by okadaic acid was observed in porcine arterial strips (Hirano et al., 1989) which is similar to the elevation of $[Ca^{2+}]i$ by the toxin of *P. patelliferum* in both synaptosomes (Paper III) and clonal anterior pituitary cells (Paper V). Finally, okadaic acid was shown to inhibit the uptake of GABA but not that of dopamine in rat brain synaptosomes (Tian et al., 1994), suggesting that the GABA transporter may be regulated by phosphorylation. It was therefore of interest to investigate the possible presence of phosphatase-inhibitors in the extract of *P. patelliferum*.

The preliminary studies in our laboratory showed that the extract of *P. patelliferum* increased the phosphorylation level of synaptosomal proteins phosphorylated by endogenous Ca^{2+}-dependent protein kinases (Figure 4) (Meldahl, unpublished data). At a concentration of purified algal extract corresponding to 100 000 cells/ml, a significant increase in the amount of phosphorylated proteins was observed both after 2 min and after 30 min.

This effect could be interpreted as a disturbance in the balance of protein phosphorylation, i. e. the balance between phosphorylation by protein kinases and dephosphorylation by protein phosphatases. Compared to the effect of okadaic acid, the results strongly indicate the presence of a phosphatase inhibitory activity in the extract of *P. patelliferum*. It is interesting to note that this concentration of purified algal extract (100 000 cells/ml) is in the same range as the concentrations needed to inhibit the uptake of L-glutamate, depolarise the plasma membrane, and increase the permeability of synaptosomes to Na^+, Ca^{2+} and K^+ (Paper III). Similar effects on the phosphorylation of synaptosomal proteins have been observed also for the toxic extracts of other potentially ichthyotoxic algae such as *P. parvum*, Gyrodinium aureolum, *C. polylepis* and *C. leadbeateri* (Meldahl; I. Walaas and A. C. Henriksen, University of Oslo; unpublished observation), suggesting the presence of similar phosphatase inhibitors in these marine algae. It is difficult to speculate from the limited information of the present data whether an inhibition of phosphatase activity would be involved in the effects of the extract of *P. patelliferum*. However, given that the voltage-activated calcium channels of pituitary cells must be phosphorylated in order to open when the membrane is depolarised (Armstrong and Eckert, 1987), it is not unlikely that an inhibition of dephosphorylation by the toxic extract was involved in the observed increase in Ca^{2+}-influx in the GH_4C_1-cells (paper IV). Determination of the types

of phosphatases affected, using pure phosphatases, would be helpful for the further investigations of this interesting property of the algal extracts.



Incubation time (min):	2	30	2	30	2	30	2	30
	Control		*P. pat* extract (10^5 cells/ml)		*P. pat* extract (10^6 cells/ml)		Okadaic acid (5 μM)	

Figure 5.2 The toxic extract of P. patelliferum (P. pat extract) inhibited the dephosphorylation of endogenous phosphoproteins of synaptosomes indicating the presence of a phosphatase inhibitor in the algal extract.

Synaptosomes obtained as described in paper III were lysed by resuspension in a hypotonic solution containing 20 mM Tris-HCl, pH 7.4 and 1 mM EDTA. The synaptosomal suspension (final protein concentration 0.1 mg/ml) was incubated with the algal extract or okadaic acid for 15 min at room temperature. γ-[^{32}P]ATP was added (final concentration 3 μCi × ml^{-1}) and the phosphorylation was started by the addition of a reaction mixture containing (final concentration): 25 mM Hepes (pH 7.4), 10 mM $MgSO_4$, 1 mM EDTA, 1 mM EGTA, 1.5 mM $CaCl_2$,
0.6 μM ATP. After incubation for 2 and 30 min, aliquots (50 μl) of each sample were transferred to SDS-containing "stop solution" containing (final concentration): 3% SDS, 5 % glycerol and 60 mM Tris-HCl (pH 6.9) and 2% mercaptoethanol and a small amount of Coomassie blue dye. Aliquots of the samples (about 2 μg protein) were subjected to discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) on 12% gels (Laemmli, 1970) with 3% stacking gel. The electrophoresis was carried out at 80 V for about 5 min and then at 190 V for about 60 min. The gels were fixed, stained, destained and dried, and subjected to autoradiography as described by Ueda and Greengard (1977).

5.5 The cytolytic effect of *Prymnesium*-toxin

We found that the extract of *P. patelliferum* was clearly hemolytic to human erythrocytes (Papers I and II). In these experiments the algal extract of *P. patelliferum* was incubated with the erythrocytes at 35 °C for 45 min to allow maximum lysis at each toxin concentration. At the highest concentrations, however, total hemolysis occurred within a shorter time (1 - 5 min) (Meldahl, unpublished observations). The synaptosomes, however, were not lysed under similar incubation conditions as no significant increase in leakage of the cytoplasmic marker lactate dehydrogenase was detected (Paper III). Nevertheless, under these conditions there was a marked increase in the permeability of the synaptosomes to the ions Na^+, Ca^{2+} and K^+ as well as total membrane depolarisation (Paper III). It is possible, however (although it remains to be tested), that incubation of synaptosomes with high concentrations of algal extract for extended periods of time (> 45 min) would have resulted in leakage of LDH and cell lysis.

Many hemolytic agents such as viruses (Pasternak and Micklem, 1973), bacterial (Freer et al., 1973), and animal (Tosteson and Tosteson, 1981) toxins or the membrane attack complex of complement (Mayer, 1972) damage cells by formation of small pores or channels, through which ions and metabolites leak out. All these agents have in common the property of lysing erythrocytes by the creation of pores[(1)] in the membrane (Pasternak, 1987). However, non-erythroid cells are not lysed by these compounds, except at high concentration of agent, because they possess mechanisms for recovery and repair (Campbell and Morgan, 1985; Carney et al., 1985) which are absent in erythrocytes. For example, non-erythroid cells possess a higher number of membrane-associated ion pumps than do erythrocytes and erythrocytes are devoid of intracellular organelles and intracellular signalling. Hence, cellular damage, not cell death, is often the outcome of the action of these pore-forming agents on non-erythroid cells (Pasternak, 1984, 1987).

It has also been shown that small amounts of pore-forming agents such as the Sendai virus, may stimulate rather than damage cells, i.e. the small lesions of the plasma membrane would depolarise the cells and allow an influx of ions such as Na^+ and Ca^{2+}, actions which trigger processes like endocrine secretion or metabolic stimulation (Pasternak, 1987). At higher concentrations of agent, repair of lesions may not occur, and the excessive influx of for example Ca^{2+}, will lead to cell damage and death (Bashford et al., 1985). It was thus proposed by Bashford et al. (1985) that hemolytic agents have a sequential mode of action, featuring (i) induction of permeability to ions, (ii) induction of leakage of small metabolites and (iii) lysis due to irreversible membrane damage. It is possible that a mechanism of action similar to that described above is the reason for the differences in lytic activity of the toxic extract of *P. patelliferum* on erythrocytes and synaptosomes. A similar sequence of

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(1) Pasternak (1987) used the term "pore" in an operational, not a structural sense, implying no more than the creation of some kind of membrane imperfection such that water soluble compounds could leak across the membrane faster than they would otherwise do.

events seemed to take place in the GH_4C_1-cells after addition of the algal extract (Paper IV), where the increase in inward flux of Ca^{2+} was followed by formation of membrane protuberances and probably cell lysis after extended periods of time (30-60 min). In short, the outcome of an interaction between a hemolytic agent and a cell depends on the extent of the lesion, which is itself dependent on the nature of the affected cell. Also, it should be kept in mind that the toxic extract of *P. patelliferum* may contain several different toxins of which some may be more lytic than others.

5.6 The toxic effect on fish

As suggested already by Ulitzur and Shilo (1966), the ichthyotoxic effect of *Prymnesium* is likely to be due to a loss of the selective permeability of the gill tissue. It is today generally believed that the ichthyotoxic activity of ichthyotoxic species of marine flagellates such as *Prymnesium* and *Chrysochromulina* originate from an interaction of the algal toxins with the fish gills, most probably by a disturbance of the osmoregulatory function (Ulitzur and Shilo, 1966; Leivestad and Serigstad, 1989; Johannessen et al., 1991).

Like all vertebrates, teleost fishes maintain a constant concentration of NaCl in their body at approximately 40% of that of sea water, and the marine fish therefore face a net diffusional uptake of salt from the sea water (Maetz, 1974). The gills are the site of large sodium and chloride exchanges, and an effect on one or more of the various gill transport systems or diffusion patterns could have profound effects on the ability of the fish to osmoregulate. The surplus of monovalente ions, mainly Na^+ and Cl^-, are secreted by the chloride cells of the gills, which contain high amounts of the enzyme Na^+/K^+-ATPase (Borgatti et al., 1992). This enzyme is almost ubiquitous in the animal world. It plays a key role in Na^+-transport and serves a variety of essential functions. The enzyme complex is an electrogenic cationic pump that actively transports Na+ out of and K+ into cells through the plasma membrane. It has been demonstrated that in the gills of euryhaline fish (fish able to adapt to both fresh water (FW) and sea water (SW), like the salmonid fishes), the number of chloride cells, and thus the activity of Na^+/K^+-ATPase, are increased when FW-adapted fish are transferred to SW (Borgatti et al., 1992). This observation strongly indicates the involvement of the Na^+/K^+-ATPase in the excretion of Na^+ from the branchial cells.

In a preliminary study (Dall-Larsen, 1993) it was found that the Na^+/K^+-ATPase activity of a vesicle preparation of the gills of SW adapted salmon was inhibited by a commercial preparation of the toxin of *Prymnesium* isolated as described by Ulitzur and Shilo (1970). The enzyme activity was inhibited by about 20% at a toxin concentration of 12 hemolytic

units/ml[(2)]. Moreover, about 80% inhibition of the vacuolar H^+-ATPase of this gill preparation was observed using only 2 hemolyic units/ml of the *Prymnesium*-toxin.

In contrast to the results of Dall-Larsen (1993), we found no significant inhibition of the Na^+/K^+-ATPase of the synaptosomal plasma membrane using amounts of the extract of *P. patelliferum* corresponding to about 20 hemolytic units (Paper III). Nor was the vacuolar H^+-ATPase of the synaptic vesicles inhibited in the presence of the toxic extract (Meldahl, unpublished observations). The reason for the discrepancy between these results is not clear, but there are two points that may be questioned. First, it is possible that the two toxic extracts differed in the composition of toxins, and that the component giving rise to the ATPase inhibition is less pronounced in the toxic extract of *P. patelliferum* than in *P. parvum*. Second, since the toxins of *Prymnesium* are lipophilic, differences in the membrane composition of the fish-gill vesicle preparation and the nerve cell preparations, especially with regard to the lipid:protein ratio, may be of importance for the different effects of the two toxic extracts. It would be of interest to study the effect of the extract of *P. patelliferum* on the fish gill preparation.

Given the marked effect of the toxin of *P. patelliferum* on the Na^+-permeability of the synaptosomal membrane (Paper III) it may be reasoned that a similar effect could occur in the fish gills, thereby interfering with the ion regulatory ability of the gills, which may finally lead to death. Moreover, based on the increased permeability to Ca^{2+} of the nerve cell membranes in the presence of the algal extract (paper III and IV) it is possible that the algal toxin is also able to increase the permeability to Ca^{2+} across the ion-transporting cells of the gills (chloride cells). This could cause critical disturbances of the cellular Ca^{2+} homeostasis in the fish. Such an effect could explain the "activation" of ichthyotoxicity of *Prymnesium* by high concentrations of Ca^{2+} in the water reported by Yariv and Hestrin (1961) (see also section 1.3). The apparent activation of voltage activated Ca^{2+}-channels by the toxic extract of *P. patelliferum* is, however, not of importance for the toxic effect on the fish gills. Voltage-gated Ca^{2+}-channels are not likely to be present in the fish gills as no effect on Ca^{2+}-influx was observed by blockers of the L-type Ca^{2+}-channel (Perry and Flik, 1988) or by depolarisation with 60 mM KCl (Flik and Verbost, 1993).

5.7 The usefulness of synaptosomes as a basis of a test method for ichthyotoxic algae

In the present study we have investigated the effects of a toxic extract of *P. patelliferum* on several membrane functions of rat brain synaptosomes. Synaptosomes were selected because these are particles with several well established membrane properties that can be

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(2) One hemolytic unit was defined as the amount of algal toxin that caused 50% lysis of 1 ml of a 0.3% human red blood cell suspension. In Paper II one hemolytic unit corresponded to 49 000 *P. patelliferum* cells.

analysed. Due to the high sensitivity, the method for determination of high affinity uptake of L-glutamate into synaptosomes has formed the basis of a method to detect the presence of toxins in natural water samples during algal blooms of this and related potentially ichthyotoxic algae (Meldahl and Fonnum, 1991).

The synaptosomes can survive in isolation for several hours, maintaining plasma and mitochondrial membrane potentials of some 60 mV and 150 mV, respectively (Scott and Nicholls, 1980), high ATP/ADP ratios (Kauppinen and Nicholls, 1986) and cytoplasmic free Ca^{2+}-concentrations in the submicromolar concentrations (Hansford and Castro, 1985). The isolation procedure is simple and requires only basic laboratory equipment. The so-called synaptosome-test (i.e. determination of inhibition of the uptake of L-glutamate into synaptosomes) is rapid and simple to perform. The toxic activity of samples of natural algal water from the affected area should be extracted as soon as possible according to the method for extraction of the *Prymnesium* toxin. When dissolved in methanol, the algal extract can be stored for a long time (> one year) at -20 °C without loss of activity (Meldahl, unpublished results). This test method has been applied with success for the verification of the presence of toxin during blooms of *P. parvum*/*P. patelliferum*, *C. polylepis* and *C. leadbeateri* occurring in the Norwegian coastal area in the years 1991-1994. The method is more sensitive than the traditional method for determination of hemolytic activity of algal extracts (Paper I and II). Moreover, like the method for determination of hemolytic activity, the synaptosome-test is sensitive to the increase in toxicity of *P. patelliferum* taking place during growth with limited amounts of phosphorus (Paper I).

An alternative sensitive method for detection of the ichthyotoxic algae is by direct determination of the toxicity of the algal water to larvae of the brine shrimp Artemia salina (Edvardsen, 1993). The so-called Artemia-test was about as sensitive as the synaptosome-test (Paper II). However, relatively long time is required to prepare the Artemia-larvae (three days), and water samples that have to bee stored during this time may change in toxicity.

Altogether, the synaptosomes constitute a useful preparation for rapid detection of the toxic activity as well as for detailed studies of the mechanisms of action of algal toxins. When the main site of action of different algal toxins has been elucidated, it may be possible to tell by the effect on the synaptosomes which alga is present in the toxic bloom.

6 CONCLUSIONS AND FURTHER PERSPECTIVES

A sensitive test method for detection of potentially ichthyotoxic species of the marine flagellates *Prymnesium* and *Chrysochromulina* has been developed. This test method determines the inhibitory activity of the lipid extract of the algae on the Na^+-dependent uptake systems for neurotransmitter substances in synaptosomes isolated from rat brain (Papers I and II).

Toxins of *P. patelliferum*, *P. parvum*, and *C. polylepis* were lethal to the brine shrimp Artemia salina, were hemolytic to human red blood cells and had similar inhibitory effects on the different transport systems for neurotransmitters in synaptosomes and synaptic vesicles of rat brain. These similarities in toxic effects indicate that these related algae may produce the same family of toxins (Paper II).

The toxic extract of *P. patelliferum* increased the permeability of synaptosomes to Na^+, K^+ and Ca^{2+} and depolarised the synaptosomal membrane (Paper III). The effect on the Na^+-permeability was predominant and may be of major importance for the inhibitory effect on the Na^+-dependent neurotransmitter uptake in synaptosomes (Paper III).

The results indicate that toxin present in the extract of *P. patelliferum* may induce influx of Ca^{2+} through voltage-dependent Ca^{2+}-channels of the nerve cell membrane. This was clearly demonstrated in pituitary cells by the increase in voltage dependent Ca^{2+}-currents as well as by the increase in the intracellular Ca^{2+}-concentration and release of prolactin, effects that were all sensitive to inhibition by the blockers of the voltage-sensitive Ca^{2+}-channels, verapamil or D600 (Paper IV).

It was shown that the algal extract potentiated the Ca^{2+}-dependent release of acetylcholine in bronchial smooth muscle induced by depolarisation with high concentration of K^+. This effect was partly inhibited by classical Ca^{2+}-channel blockers, further supporting a possible effect of the algal extract on voltage-activated Ca^{2+}-channels (Paper V). The toxic extract also induced efflux of acetylcholine under resting conditions, an effect which was not dependent of $Ca2^+$. These results indicate that more than one toxic entity are present in the crude extract of *P. patelliferum*.

On the basis of the observed increase in the ion permeability of the nerve cell plasma membrane by the toxic extract of *P. patelliferum*, it is possible that a similar action on the fish gills could lead to fish death due to a disturbance of their ion regulatory abilities.

The multiple toxic effects of the lipid extract of *Prymnesium patelliferum* demonstrated in the present investigations indicate the presence of several toxic compounds. Isolation of the toxic substances of *P. patelliferum* and elucidation of their chemical structures will facilitate the studies of their exact mechanism of action. Moreover, it will open the possibility for studies of the algal biosynthesis of these compounds as well as studies of environmental factors that may be important for the stimulation of the toxin-production. Based on the

close familiar relationship between the potentially ichthyotoxic algae *P. patelliferum*, *P. parvum*, *C. polylepis* and *C. leadbeateri*, it is likely that much of the knowledge of the toxin production in one of these (e.g. *P. patelliferum*) can apply also to the other related species.

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PAPERS I - V

PAPER I

Meldahl, A.-S. and Fonnum, F. (1993) Effect of toxin of *Prymnesium patelliferum* on neurotransmitter transport mechanisms. Development of a sensitive test method. *J. Toxicol. Environ. Health,* **38,** 57-67.

EFFECT OF TOXIN OF *PRYMNESIUM PATELLIFERUM* ON NEUROTRANSMITTER TRANSPORT MECHANISMS: DEVELOPMENT OF A SENSITIVE TEST METHOD

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A crude extract of the ichthyotoxic phytoflagellate Prymnesium patelliferum *strongly inhibited the uptake of neurotransmitters into isolated nerve endings (synaptosomes) and synaptic vesicles. These systems were about 100 and 10 times more sensitive toward the algal toxin, respectively, than a standard hemolysis assay often used for testing the toxicity of* Prymnesium *spp. and other ichthyotoxic algae.* Prymnesium patelliferum *grown in phosphorus-deficient (−P) medium was about five times more active than when grown in phosphorus sufficient (+P) medium. The inhibition ratio between the high-affinity synaptosomal uptake of L-glutamate and γ-aminobyturic acid (GABA) was 1/2.7 for the −P culture and 1/1.9 for the +P culture. The inhibition ratios for the low-affinity vesicular uptake of L-glutamate, GABA, and dopamine (DA) were 1/5.8/0.3 and 1/1.7/0.2, respectively. The synaptosomal transport of L-glutamate is a rapid, simple, and sensitive test method for toxicity determination of* Prymnesium *spp. and will be a useful tool in the further isolation and purification of the toxic principles of this and other related algae. It is suggested that the toxin interferes with ion channels or acts as an ionopore.*

INTRODUCTION

The toxin-forming phytoflagellates *Prymnesium parvum* Carter and *P. patelliferum* Green (Prymnesiophyceae) are widespread in many regions of the world, and their toxin principle is notorious for its biological activity in nature as causing death to fish populations in brackish water (Green et al., 1982; Shilo, 1982). In July–August 1989, 1990, and 1991, *Prymnesium* spp. (*P. parvum* and *P. patelliferum*) in blooms in the coastal waters of southwestern Norway have caused great damage to the aquaculture activity.

Prymnesium parvum and *P. patelliferum* are morphologically very similar, and species of *Prymnesium* have often been identified as *P. par-*

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vum on the basis of light microscopy only. Differentiation between the two species is possible only after investigation of the body scales in electron microscope. Both species are toxic to fish and are found in brackish water (Larsen and Moestrup, 1989). Since the information on *P. patelliferum* is quite new, it may be that the organism responsible for some of the sudden mass fish mortalities in different areas has been this species and not *P. parvum*. Considering the very close relationship between the two species, their toxins might be similar or the same. To our knowledge there has been no published work on the toxin of *P. patelliferum* since it was distinguished from *P. parvum*. Thus, the present work was performed and results are discussed on the basis of what is known from the literature on *P. parvum*.

The salmon and the rainbow trout exposed to a toxic bloom of *Prymnesium* spp. die within a short time, after spasmodic movements. These signs suggest neurotoxic effects (Parnas, 1963; Parnas and Abbott, 1965); however, in vitro experiments also show hemolytic activity (Martin and Padilla, 1971; Paster, 1973; Yariv and Hestrin, 1961) and cytotoxic effects on various cells such as Ehrlich ascites cells (Dafni, 1971; Dafni and Shilo, 1966), HeLa cells, and normal liver and amnion cells in tissue culture (Shilo and Rosenberger, 1960). Studies on the action of *Prymnesium* toxin have been carried out using liposomes and black lipid membranes (Imai and Inoue, 1974; Moran and Ilani, 1974), indicating that the toxin interacts with membrane sterols forming negatively charged aqueous pores. The neurotoxic effect on various muscle preparations included both excitation and block of contractions induced by smooth-muscle stimulants (Parnas and Abbott, 1965).

The chemical structure of the toxin has not been elucidated fully, and the conclusions from the studies are contradictory and confusing. Ulizur and Shilo (1970) separated six hemolytically active components and the purified complex was described as a proteolipid, whereas Paster (1973) presumed the toxin to be a glycolipid. Isolation and structural analysis of the major hemolysin of *P. parvum* by Kozakai et al. (1982) revealed a mixture of two digalactosyl monoglycerides, differing only in the degree of saturation of the 3-position fatty acid chain.

In the search for a simple and sensitive test method for detection of ichthyotoxin in the waters threatened by toxic blooms of *Prymnesium*, the toxin was tested on two well-established membrane transport systems for neurotransmitters. One preparation is a fraction of isolated nerve cell terminals (synaptosomes) that exhibit high-affinity uptake of neurotransmitters over the membrane. This transport is dependent on external sodium and a sodium gradient maintained by Na^+,K^+-ATPase activity (Kanner, 1978; Kanner and Sharon, 1978). The other nerve cell fraction consists of synaptic vesicles, organelles storing the neurotransmitters inside the nerve cell terminals. The low-affinity vesicular uptake of transmitter does not require sodium and is driven by an electrochemi-

cal proton gradient generated by a Mg^{2+}-ATPase (Fykse et al., 1989). Further, for both systems the uptake of different transmitters possess distinct characteristics with respect to dependency on certain ions, ion gradients, or membrane potential. Thus, neurotransmitters were chosen so as to enable us to detect specific effects by the toxin on the factors involved in the uptake mechanisms. The amino acids L-glutamate and GABA are important neurotransmitters in the central nervous system, and the high-affinity uptake of both is well characterized (Fonnum et al., 1980). The accumulation into synaptic vesicles is driven by the transvesicular proton electrochemical gradient. However, the component used (vesicular membrane potential V_v, or ΔpH) varies. Vesicular uptake of L-glutamate, GABA, and dopamine was chosen because the substrates differ in their reliance on V_v and ΔpH (Fykse and Fonnum, 1988; Kanner, 1983; Maycox et al., 1988). Also, the toxin activity on these two transport systems has been compared with the hemolytic activity in the standard hemolysis assay of Yariv and Hestrin (1961).

Measurements of hemolytic, cytotoxic, and ichthyotoxic activity have shown that the toxin production of *P. parvum* is promoted by phosphorus deficiency in the algal growth medium (Shilo, 1971). The production of neurotoxin by algae grown in a medium sufficient and deficient in phosphorus was therefore examined.

MATERIALS AND METHODS

Materials

A clone of *P. patelliferum* from a bloom in the Hylsfjord, southwestern Norway, 1989, was a gift from Professor E. Paasche, Department of Biology, University of Oslo, Norway. Male Wistar rats (150–200 g) were obtained from Møllegaard, Denmark. L-Glutamate (dipotassium salt), GABA, and ATP (disodium salt) were purchased from Sigma Chemical Co. (St. Louis, Mo.). L-[2,3-^{3}H]Glutamate (17.30 Ci/mmol), [2,3-^{3}H]GABA (40 Ci/mmol), and [2,5,6-^{3}H]dopamine (9 Ci/mmol) were obtained from New England Nuclear (Boston).

Algal Cultures

Prymnesium patelliferum was grown in 3-l Erlenmeyer flasks containing 2 l filtered and autoclaved seawater diluted to 9‰ salinity and with nutrients added as in IMR 1/2 medium (Eppley et al., 1967). For the phosphorus-starved cultures, no phosphate was added to the medium. The algae were grown for about 2 wk at 20°C under white fluorescent light with a quantum flux of 200 $\mu E\ m^{-2}\ s^{-1}$ and a 12-h light-dark cycle. The cultures were concentrated to 100 ml by ultrafiltration (Filtron Minisette, Omega membrane, 1 MDa) and stored at −20°C for 1–6 d.

Extraction

A crude toxin extract was obtained as described by Ulitzur and Shilo (1970). The algal culture was mixed with 4 volumes of methanol-chloroform (1:2). The aqueous methanol was reextracted with chloroform and the pooled chloroform fractions were evaporated to dryness with a rotavapor. The extract was dissolved in methanol and stored in the dark at 2–4°C without loss of activity until testing within 1–4 d.

Preparation of Synaptosomes and Synaptic Vesicles

Male Wistar rats (200–250 g) were killed by decapitation. The brain was quickly removed and kept on ice. Synaptosomes were prepared as described in principle by Gray and Whittaker (1962). A brain homogenate (5% w/v) was made in 0.32 *M* sucrose. The homogenate was centrifuged (800 × g, 10 min) and the supernatant further centrifuged (15,500 × g, 30 min). The resulting pellet of synaptosomes (P_2) was resuspended in 0.32 *M* sucrose to a 5% (original w/v) solution. The synaptosome preparation was used in the experiment the same day.

Synaptic vesicles were prepared as previously described (Fykse and Fonnum, 1988). The P_2 fraction obtained as already described was hypoosmotically shocked by resuspension in 10 m*M* Tris maleate (pH 7.4) and 0.1 m*M* EGTA. The solution was then centrifuged (14,000 × g, 30 min). The supernatant was laid on top of a discontinuous gradient of 0.4 *M* and 0.6 *M* sucrose solutions and centrifuged in a Contron TST 28.38 rotor (65,000 × g, 1 h). The vesicle fraction was isolated from the band containing 0.4 *M* sucrose and stored in liquid nitrogen without loss of activity.

Assay of Neurotransmitter Uptake

High-affinity uptake into synaptosomes was measured as described by Fonnum et al. (1980). Crude synaptosomal fraction (2 μl containing 2–4 μg protein) was preincubated for 15 min at 25°C with toxin extract (1–3 μl) and 0.5 ml of Tris-Krebs medium containing 15 m*M* Tris, 140 m*M* NaCl, 5 m*M* KCl, 1.2 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 1.2 m*M* Na_2HPO_4, 10 m*M* glucose, pH adjusted to 7.4, and bubbled with O_2. Substrate solution (25 μl) containing L-[^{3}H]glutamate (final concentration 97 n*M*, 17.30 Ci/mmol) or [^{3}H]GABA (final concentration 42 n*M*, 40.00 Ci/mmol) was added and the synaptosomes were incubated for 3 min at 25°C. The uptake was terminated by filtration with a solution of 0.15 *M* NaCl and bovine serum albumin (0.5 g/l) in a cell harvester (Skatron) onto a glass-fiber filtermat. Filters were dissolved in 10 ml Filter Count (Packard) and the radioactivity was determined in a Packard Tri-Carb 2200 liquid scintillation counter. Blanks contained 0.32 *M* sucrose instead of the Tris-Krebs medium and were incubated at 0°C. Each assay was carried out in triplicate. All con-

trol incubations contained the same trace concentrations of methanol, and the degree of inhibition by the algal toxin extract is expressed as percent of control.

Some filters were extracted with trichloroacetic acid to release the amino acids, and the solution was then treated with ether to remove trichloroacetic acid. High-performance liquid chromatography (HPLC) of the radioactivity taken up into the synaptosomes was performed as previously described (Lindroth and Mopper, 1979). The amino acid content in the HPLC fractions was determined by scintillation counting.

Vesicular uptake was carried out as previously described (Fykse and Fonnum, 1988). Synaptic vesicles (0.05–0.1 mg protein) were preincubated with the toxin extract for 15 min at 30°C in 0.32 *M* sucrose, 10 m*M* Tris maleate (pH 7.4), and 4 m*M* $MgCl_2$. Then this mixture (275 μl) was incubated for 3 min with 25 μl of a substrate solution containing L-[^{3}H]glutamate (final concentration 1 m*M*, 1.7 mCi/mmol), [^{3}H]GABA (final concentration 1 m*M*, 3.6 mCi/mmol) or [^{3}H]DA (final concentration 114 n*M*, 9 Ci/mmol) and ATP (final concentration 2 m*M*). Uptake was terminated by addition of 7 ml of ice-cold 0.15 *M* KCl, immediately followed by rapid filtration and washing (2 × 7 ml 0.15 *M* KCl) through a Millipore HAWP filter (diameter 25 mm, pore size 0.45 μm). Filters were dissolved in 10 ml Filter Count (Packard) and the radioactivity was determined by scintillation counting. Blanks were treated likewise but incubated at 0°C. Each assay was carried out in duplicate. All control incubations contained the same trace concentrations of methanol, and the degree of inhibition by the toxin extract is expressed as percent of control.

Hemolytic Assay

The assay was modified from Yariv and Hestrin (1961). Human blood was collected in vacuum containers containing EDTA. Fresh blood (10 μl) was washed twice with 100 μl buffered saline solution (0.13 *M* NaCl, adjusted to pH 5.0 with 0.2 *M* Na citrate), and resuspended in 2.8 ml buffered saline solution. Dilutions (200 μl) of toxin extract in methanol were added and the mixture incubated at 35°C for 45 min. Absorbance of hemoglobin was measured at 540 nm (Beckman DU-50 series spectrophotometer). Total hemolysis was obtained with substitution of 100 μl of 0.1% saline solution of saponin in the solution of resuspended erythrocytes. Assays were carried out in duplicate. Results are given as percent of total hemolysis.

Determination of ED50

The ED50 values are presented as the number of cells from which the toxin is extracted, and were calculated by a nonlinear regression program.

RESULTS

The toxin extract of *P. patelliferum* strongly inhibited the uptake of neurotransmitter substances into synaptosomes and synaptic vesicles. The degree of inhibition was compared on the basis of the ED50 values. In Figure 1 this is illustrated by the uptake of L-[^{3}H]glutamate into synaptosomes and synaptic vesicles. Figure 1 also shows that the two nerve cell preparations were about 100 and 10 times, respectively, more sensitive than the standard hemolysis assay. About 5 times more neurotoxic activity was extracted from algae grown under phosphorus-limited conditions than from algae grown under phosphorus-sufficient conditions, whereas the hemolytic activity increased about 10-fold.

The effect of the toxin extract on the different uptake systems was compared. The synaptosomal high-affinity uptake of L-glutamate was more sensitive to inhibition by the toxin extract than that of GABA. Inhibition ratios between the two substrates were 1/2.7 and 1/1.9 for toxin extract of phosphorus-sufficient and phosphorus-deficient cultures, respectively (Table 1). As for synaptic vesicles, the degree of inhibition of the uptake decreased in the order dopamine, L-glutamate, GABA. The inhibition ratios between the vesicular uptake of L-glutamate, GABA, and dopamine for the toxin extract of the +P and −P cultures were 1/1.7/0.2 and 1/5.8/0.3, respectively.

The HPLC analysis of extracts of synaptosomes (P_2 fraction) subjected to L-glutamate uptake showed no metabolism of glutamate under the present test conditions, and almost all the radioactivity was found in the L-glutamate peak.

DISCUSSION

Preparations of nerve cell membranes are sensitive structures suitable for testing toxins. Synaptosomes are bioenergetically autonomous, and synaptosomes continue to function for at least 6 h after preparation when supplied in ice-cold sucrose (McMahon and Nicholls, 1991). The toxin extract of *P. patelliferum* appeared to be more inhibitory to the high affinity uptake of L-glutamate than to that of GABA (Table 1). This result suggests a specific nature of the toxin, since the mechanism for the high-affinity uptake differs for various transmitters. In addition to the sodium dependency and the sodium gradient maintained by the Na^+,K^+-ATPase, uptake of GABA requires low concentrations of external chloride (Kanner and Sharon, 1978), whereas the uptake of L-glutamate is absolutely dependent on internal potassium (Roskoski et al., 1981). Moreover, the sodium dependency is less pronounced for the uptake of L-glutamate than for the GABA uptake (Fonnum et al., 1980). Since the effect on the Na^+,K^+-ATPase was insignificant (results not shown), the inhibition is probably due to an interference with ion channels. A direct effect on the

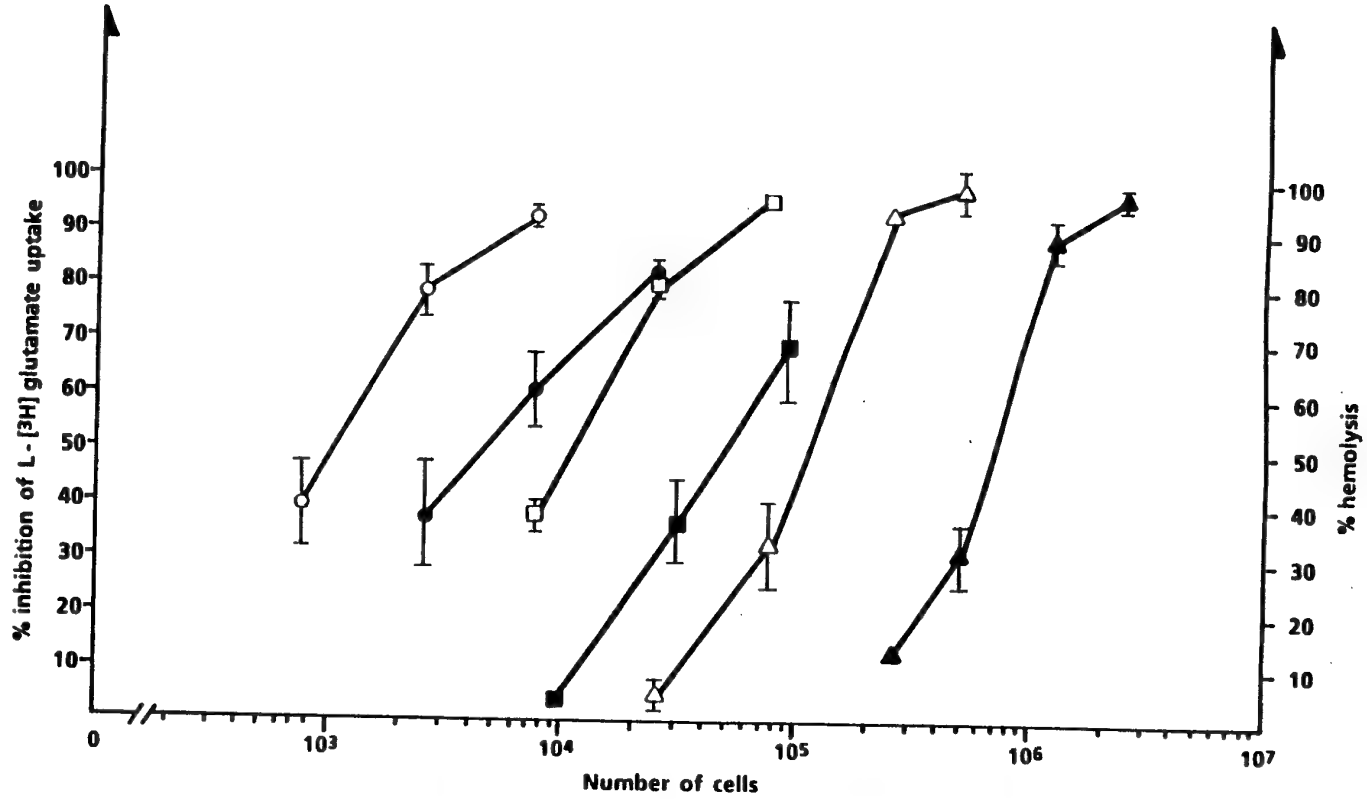

FIGURE 1. Dose-response relationship for toxin extract from *Prymnesium patelliferum* on the transport of L-[^{3}H]glutamate in synaptosomes (circles) and synaptic vesicles (squares) and on lysis of erythrocytes (triangles). The dose is the number of cells from which the toxin was extracted. Each point is the mean ± SEM of extract from three to four different algal cultures. Open symbols, toxin extract of phosphorus-deficient algae; solid symbols, toxin extract of phosphorus-sufficient algae.

TABLE 1. Effect of Toxin Extract from *Prymnesium patelliferum* on the Uptake of Neurotransmitters into Synaptosomes and Synaptic Vesicles, and on Erythrocytes

	Synaptosomal uptake			Vesicular uptake				
Algal culture	[^{3}H]glu	[^{3}H]GABA	[^{3}H]glu: [^{3}H]GABA	[^{3}H]glu	[^{3}H]GABA	[^{3}H]DA	[^{3}H]glu:[^{3}H]GABA: [^{3}H]DA	Hemolysis
−P	852 ± 66	1580 ± 125[b]	1:1.9	10,043 ± 792	58,293 ± 5430[a]	3150 ± 203	1:5.8:0.3	73,020 ± 7560
+P	3013 ± 625	8142 ± 276	1:2.7	72,637 ± 7846	125,213 ± 23,066[a,c]	16,937 ± 2278	1:1.7:0.2	649,180 ± 55,230

Note. The numbers are ED50 values and indicate the number of cells from which the toxin was extracted. Each value is the mean ± SEM of the ED50 value obtained from toxin extract of three to five different algal cultures. −P, Toxin extract of phosphorus-limited cultures; +P, toxin extract of phosphorus-sufficient cultures. The difference in the activity of the toxin extract of algae grown in phosphorus-deficient and phosphorus-sufficient medium was significant for all determinations with $p < .05$, by Student's *t*-test, except for the vesicular GABA uptake, [a]$p < .09$. The uptake of the other neurotransmitters differed from that of L-glutamate by $p < .05$ except for [b]$p < .07$ and [c]not significant (Student's *t*-test).

transport site is unlikely since there is presumably no structural similarity between the toxin and all three transmitter substances.

Also, for the vesicular low-affinity uptake there was a clear difference in the degree of inhibition between the uptakes of the various neurotransmitters (Table 1). The inhibition could be due to an effect on one or more of the factors essential for the vesicular uptake. The specific properties of the uptakes are as follows: L-Glutamate uptake is optimal when V_v is high and ΔpH is low, and some chloride is also essential (Fykse et al., 1989; Hell et al., 1990; Naito and Ueda, 1985). GABA uptake is believed to be equally dependent on both V_v and ΔpH, and no Cl^- is required (Fykse et al., 1989; Fykse and Fonnum, 1991), whereas the ΔpH component is the most important factor for the uptake of dopamine (Njus et al., 1986). The uptake of dopamine was most sensitive to inhibition by the toxin extract, and thus the toxin could affect the proton gradient. However, this theory is not in accordance with the observed relative inhibition activity on the uptake of GABA and L-glutamate, since the uptake of L-glutamate is optimal when V_v is high and ΔpH is low. There was no effect on the vesicular Mg^{2+}-ATPase, but a decrease in the vesicular ΔpH has been observed (results not shown). The effect on V_v remains to be tested.

In accordance with what is found for *P. parvum* (Dafni et al., 1972; Shilo, 1971), an increase in the hemolytic activity of about 10-fold was observed in phosphorus starved cells of *P. patelliferum*. The present work showed an increase also in the production of the neurotoxic compound upon phosphorus starvation, although only about fivefold. This result could indicate that the principles responsible for the various toxic effects (ichthyotoxic, hemolytic, cytotoxic, neurotoxic) may be structurally similar but not necessarily identical. The inhibition ratios between the synaptosomal uptake of L-glutamate and GABA and between the vesicular uptake of L-glutamate, GABA, and DA differed for toxin extract of phosphorus-deficient and phosphorus-sufficient cultures. For the synaptosomal uptake the inhibition ratios between L-glutamate and GABA were 1/1.9 and 1/2.7 for the toxin of the two algal cultures, respectively. On the basis of the separate experiments these results are not significantly different (Table 1), but we feel that they should not be overlooked. As for the vesicles, the inhibition ratio between the uptake of L-glutamate and GABA for toxin of the phosphorus-deficient (1/5.8) and phosphorus-sufficient (1/1.7) cultures seem to be significantly different. The reason for this difference is uncertain at this stage. Further purification and isolation of the toxic components are needed in order to tell whether the differences in the toxic action depending on the algal growth conditions are due to production of a toxin complex with a different composition of the active components. The fact that phosphorus is a critical factor in the toxin production of this and related algae is supported by measurements that have indicated phosphate deficiency during toxic blooms of *Prymne-*

sium spp. (*P. parvum* and/or *P. patelliferum*) and *Chrysochromulina polylepis* (Johnsen et al., 1989).

Purification and isolation of the toxic compound(s) are required to obtain more information on its (their) specific action(s). The synaptosome test method is rapid, simple, and sensitive and will be of great value during the separation of the toxic activities. It has already been used in identifying the toxin present in the outburst of *Prymnesium* spp. in Hylsfjord, Rogaland, 1991. The assumption that the inhibition of neurotransmitter transport involves an action on ion channels remains to be explored.

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PAPER II

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TOXICITY OF FOUR POTENTIALLY ICHTHYOTOXIC MARINE PHYTOFLAGELLATES DETERMINED BY FOUR DIFFERENT TEST METHODS

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The toxicity of the marine phytoflagellates Prymnesium parvum, Prymnesium patelliferum, Chrysochromulina polylepis, *and* Chrysochromulina leadbeateri *isolated from ichthyotoxic blooms in Norwegian coastal waters was compared using four different test methods developed for the detection of toxins produced by these species. The test methods were (1) lethality to the crustacean* Artemia salina *exposed to living algae, (2) hemolytic activity (lysis of human erythrocytes) by crude algal lipid extracts, and inhibition of the uptake of the neurotransmitters L-glutamate and γ-aminobutyric acid (GABA) into (3) synaptosomes and (4) synaptic vesicles of rat brain by crude algal lipid extracts. All test methods indicated different levels of toxicity among the algal species.* Prymnesium parvum, P. patelliferum, *and* C. polylepis *were toxic as determined by all four test methods. The cultured strain of* C. leadbeateri, *although isolated from a toxic algal bloom, appeared nontoxic by the methods used here, and served as a negative control. The hemolytic activity of* P. parvum *extract was about nine times higher than that of* P. patelliferum *extract, whereas the activity was only two to three times higher using the other three methods.* Chrysochromulina polylepis *had higher toxic activity than* P. patelliferum *except for less inhibitory effect on synaptosomes. The inhibition of synaptosomal and vesicular neurotransmitter uptake systems by extracts of* P. parvum, P. patelliferum, *and* C. polylepis *appeared to be due to similar mechanisms of action, since similar inhibition ratios between the uptake of L-glutamate and GABA were obtained in both synaptosomes and synaptic vesicles. We suggest that* P. parvum, P. patelliferum, *and* C. polylepis *produce a set of similar toxins and that the relative amounts of each toxin vary among the three species.*

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Blooms of the prymnesiophycean flagellates *Prymnesium parvum* Carter, *Prymnesium patelliferum* Green, Hibberd et Pienaar, *Chrysochromulina polylepis* Manton et Parke, and *Chrysochromulina leadbeateri* Estep, Davis, Hargraves et Sieburth, have been associated with fish mortality in Norwegian waters in recent years. The first registered toxic bloom of a *Chrysochromulina* species occurred in Skagerrak and Kattegat in May–June 1988, when *C. polylepis* selectively decimated benthic organisms and farmed fish (Rosenberg et al., 1988; Dahl et al., 1989). In May and June 1991, *C. leadbeateri* caused the death of hundreds of tons of salmon and rainbow trout in fish farms in northern Norway (Aune et al., 1992; Eikrem & Throndsen, 1993). *Prymnesium parvum* and *P. patelliferum* have coexisted in the annual toxic blooms in the brackish fjords of southwestern Norway and have caused a variable degree of mortality of farmed fish during the months July and August in the period of 1989–1993 (Kaartvedt et al., 1991; Eikrem & Throndsen, 1993).

Of these marine flagellates, *P. parvum* has been most extensively studied. This alga has both ichthyotoxic and cytotoxic activity. The ichthyotoxic activity appears to be due to a loss of the selective cell permeability in the fish gills (Ulitzur & Shilo, 1966), and the cytotoxic activity likewise appears to be mediated by membrane destabilization (for review see Paster, 1973). When exposed to *P. parvum* toxin, red blood cells, various mammalian cells, and bacteria underwent morphological changes that often led to lysis. Moreover, various neurotoxic effects have been observed, such as induction of contraction of smooth muscle, and postsynaptic blockage of the neuromuscular junction. It is not known whether the various biological effects were due to the same or different compounds produced by *P. parvum*. From thin-layer chromatography of a purified methanol extract of *P. parvum* it was evident, however, that this alga contains at least six hemolytic compounds (Ulitzur & Shilo, 1970). The quantitatively dominating hemolytic compound was found to be a mixture of two similar glycolipids (Kozakai et al., 1982). Recently, the same research group isolated and partly determined the structure of two closely related polyhydroxy-polyene-polyether compounds (Igarashi et al., 1993). Glycolipids similar to those of *P. parvum* have been isolated from *C. polylepis* (Yasumoto et al., 1990). Crude lipid extracts of the latter alga were hepatotoxic (Underdahl et al., 1989), hemolytic (Edvardsen et al., 1990), and inhibited the uptake of neurotransmitters into synaptosomes (Meldahl et al., 1993). Similar effects have been demonstrated for crude lipid extract of natural samples from the toxic bloom of *C. leadbeateri* (Aune et al., 1992; Meldahl & Fonnum, 1991) and for *P. patelliferum* cultures (Meldahl and Fonnum, 1993). The toxic compounds in *C. leadbeateri* or *P. patelliferum* have not been chemically characterized.

In the present work we have compared the toxic activities of axenic monocultures of *P. parvum*, *P. patelliferum*, *C. polylepis*, and *C. leadbeateri* isolated from toxic blooms. Four different test methods that detect an effect by toxin on biological membranes were used. The toxicity of the living algae

was determined as lethality to larvae of the crustacean *Artemia salina* (*Artemia* bioassay). Crude lipid extracts of the algal cultures were tested for hemolytic activity to human red blood cells (hemolysis test) and for inhibition of the uptake of the neurotransmitters L-glutamate and γ-aminobutyric acid (GABA) into isolated nerve terminal plasma membranes, synaptosomes, and intracellular storage organelles, synaptic vesicles, of rat brain (synaptosome test and vesicle test, respectively). The membranes of these nerve cell preparations, synaptosomes, and synaptic vesicles are more developed than most other membranes, and therefore constitute interesting test objects for toxins acting on biological membranes. The neurotransmitter transport systems of synaptosomes are coupled to a sodium gradient maintained by a Na^+/K^+-ATPase (Kanner, 1978; Kanner & Sharon, 1978), whereas the transport systems of the synaptic vesicles are driven by an electrochemical proton gradient generated by an Mg^{2+}-activated H^+-ATPase (Fykse et al., 1989). The synaptosomal transport systems differ in their requirement for additional ions, potassium being required for L-glutamate transport and chloride for GABA transport (for review see Kanner, 1983). In synaptic vesicles the two uptake systems differ in that L-glutamate uptake requires a large membrane potential and a small pH gradient (Maycox et al., 1988), whereas GABA uptake is equally dependent on both the membrane potential and the pH gradient (Hell et al., 1990). In this study it is shown that all four test methods can be used for testing the toxic activity of species of potentially ichthyotoxic Prymnesiophyceae, although with difference in sensitivity. Using these test methods we were able also to discuss some similarities in the mechanism of action and in the composition of toxic substances of the potentially ichthyotoxic marine algae.

MATERIALS AND METHODS

Algal Strains

Axenic strains of *P. parvum*, *P. patelliferum*, *C. polylepis*, and *C. leadbeateri* were used. The cultures originated from samples collected from toxic algal blooms: *P. parvum*, strain K-0081BF, 1985.05.29, Fladsøen, Denmark; *P. patelliferum*, strain N, 1989.08.03, Hylsfjorden, Norway; *C. polylepis*, clone S, 1988.06.06, Skagerrak, Norway; and *C. leadbeateri*, strain TJE-1, 1991.05.30, Vestfjorden, Norway.

Chemicals

L-Glutamate (dipotassium salt), γ-aminobutyric acid (GABA), and ATP (disodium salt) were purchased from Sigma Chemical Co. (St. Louis, Mo.). L-[2,3-^{3}H]Glutamate (17.30 Ci mmol^{-1}) and L-[2,3-^{3}H]GABA (40 Ci mmol^{-1}) were obtained from New England Nuclear (Boston). Saponin (white) was obtained from BDH Chemicals Ltd. (Poole, England).

Growth and Extraction of Algal Cultures

Prymnesium parvum, P. patelliferum, C. polylepis, and *C. leadbeateri* were grown in filtered and autoclaved seawater diluted to optimal salinity (9‰ salinity for the *Prymnesium* species and 25‰ salinity for the *Chrysochromulina* species) with nutrients added as in IMR ½ medium (Eppley et al., 1967), but without phosphorus, and supplemented with 10^{-8} *M* selenite (Dahl et al., 1989). Phosphorus limitation of algal cultures was shown to promote toxin production in *P. parvum* (Shilo, 1971), *P. patelliferum* (Meldahl & Fonnum, 1993), and *C. polylepis* (Edvardsen et al., 1990). Four batch cultures of each species were grown in 3-L Erlenmeyer flasks with gentle aeration under white fluorescent light, with a photon fluence rate of 130 μmol photons m^{-2} s^{-1} and a 12 h-12 h light-dark cycle. The growth temperature was 20°C for the *Prymnesium* species and 15°C for the *Chrysochromulina* species. At the end of the exponential growth phase (cell density 1–3 × 10^5 algal cells ml^{-1}) the cultures were concentrated by ultrafiltration (Filtron Minisette, Omega membrane, 1 MD) and stored at –80°C for up to 4 wk. The cell concentration was determined in an electronic particle counter (Coulter Electronics Ltd., model D). Crude toxin extracts were obtained by extraction as described by Ulitzur and Shilo (1970). One volume of the concentrated algal culture was mixed with 4 volumes of methanol-chloroform (1 : 2), and the chloroform fraction was evaporated to dryness in a rotavapor. The dry extracts were dissolved in methanol and stored in darkness at –20°C for up to 4 wk without loss of activity (unpublished data).

Artemia Bioassay

Toxicity tests of fresh algal cultures were performed on the same day as the cultures were concentrated. Larvae of the crustacean *Artemia salina* (Creasel, Deinze, Belgium) were used following the protocol of the *Artemia* Reference Center (Ghent University, Belgium) as described by Edvardsen (1993). Exponentially growing algal cultures were diluted to 5 different concentrations with autoclaved seawater to a final salinity of 25‰. Forty stage 2 or 3 *A. salina* nauplii were exposed to each cell density for 24 h at 25°C in darkness. Death was defined as nonmotility for more than 10 s. Spontaneous mortality on seawater of 25‰ salinity was less than 1%.

Hemolysis Assay

Hemolytic activity was determined colorimetrically using the method of Yariv and Hestrin (1961) as earlier described (Meldahl & Fonnum, 1993). Five different concentrations of algal extract in methanol were added to suspensions of human erythrocytes in saline buffer, pH 5.5 (0.3% v/v of original blood), and the mixtures were incubated at 35°C for 45 min. The suspensions were centrifuged (3 min, 2000 rpm, Heraeus Christ Biofuge A), and the absorbance of the supernatant was measured at 540 nm (Beckman DU-50 series spectrophotometer). Methanol (7%) had minor effects on the red blood

cells. All assays were carried out in duplicate. Results were given as percent of total hemolysis obtained with saponin (30 μg ml^{-1}).

Uptake of L-Glutamate and GABA into Synaptosomes

Male Wistar rats (200–250 g, Møllegaard Breeding Laboratories, Denmark) were killed by decapitation. The brains were quickly removed and kept on ice. Crude synaptosomal fraction (P_2) was prepared from whole-brain homogenates as described by Gray and Whittaker (1962). The pellet of crude synaptosomes was resuspended in 0.32 *M* sucrose to a 5% solution (original w/v) and used in the experiment the same day.

High-affinity uptake of L-[^{3}H]glutamate and [^{3}H]GABA into synaptosomes was determined by the method of Fonnum et al. (1980) as developed by Meldahl and Fonnum (1993). Briefly, synaptosomes (4–8 μg protein ml^{-1}) in Tris-Krebs buffer, pH 7.4, were preincubated with the algal extracts for 15 min at 25°C, and uptake of L-[^{3}H]glutamate (97 n*M*, 17.30 Ci mmol^{-1}) or [^{3}H]GABA (42 n*M*, 40.00 Ci mmol^{-1}) took place for 3 min at 25°C. The uptake was terminated by filtration and washing with 0.15 *M* NaCl containing bovine serum albumin (0.5 mg ml^{-1}). Controls containing the same concentrations of methanol as the samples with algal extracts (0.2–0.6%) showed no effect on the synaptosomal neurotransmitter uptake. Each assay was carried out in triplicate. The degree of inhibition by the algal extracts was expressed as percent of control after subtraction of values of blank samples (synaptosomes in 0.32 *M* sucrose, incubated at 0°C).

Uptake of L-Glutamate and GABA into Synaptic Vesicles

Synaptic vesicles were prepared from the P_2 fraction of whole-brain homogenate by the method of Whittaker et al. (1964) modified as described by Fykse and Fonnum (1988).

Vesicular uptake was carried out as previously described (Fykse & Fonnum, 1988). Synaptic vesicles (0.15–0.3 mg protein/ml) were preincubated with the algal extract for 15 min at 30°C in 0.32 *M* sucrose, 10 m*M* Tris-maleate (pH 7.4), and 4 m*M* $MgCl_2$. Substrate containing ATP (2 m*M*) and L-[^{3}H]glutamate (1 m*M*, 1.7 mCi mmol^{-1}) or [^{3}H]GABA (1 m*M*, 3.6 mCi mmol^{-1}) was added, and the neurotransmitter uptake took place for 3 min at 30°C. The uptake was terminated by addition of ice-cold 0.15 *M* KCl followed by filtration and washing. Each assay was performed in duplicate. Controls with methanol in the same concentrations as the samples with algal extracts (0.3–1.0%) had no effect on the vesicular uptake. The degree of inhibition by the algal extracts was expressed as percent of control after subtraction of values of blank samples, which were treated as the samples, but incubated at 0°C.

Statistical Analysis

Percentage mortality of *A. salina* by fresh algae, percentage hemolysis, or percentage inhibition of neurotransmitter uptake in synaptosomes or

synaptic vesicles by algal extracts, different from 0% and 100%, were probit-transformed and plotted against log cell concentration (Hewlett & Plackett, 1979). Linear regression lines were based on the results of four parallel algal cultures. The algal concentrations causing 50% mortality of *A. Salina* (LC50), 50% hemolysis (EC50), and 50% inhibition of neurotransmitter uptake in snaptosomes or synaptic vesicles (IC50) were determined from the regression line for each algal species. Probit unit 5 corresponds to 50% effect. The concentration of algal extract is given as the corresponding number of cells extracted.

RESULTS

Prymnesium parvum, P. patelliferum, and *C. polylepis* were found to be toxic by all four test methods, and dose-response relationships were established (Figure 1). Whole cultures of the species were clearly toxic to nauplii of *A. salina,* the toxicity decreasing in the order *P. parvum, C. polylepis, P. patelliferum,* with LC50 values of 1100, 1600, and 2400 cells ml^{-1}, respectively (Table 1). Cultures of *C. leadbeateri* appeared nontoxic to *A. salina,* as no effect was observed by algal densities up to 100,000 cells ml^{-1}, nor with a concentrated culture of cell density 1.5×10^6 cells ml^{-1} (not shown).

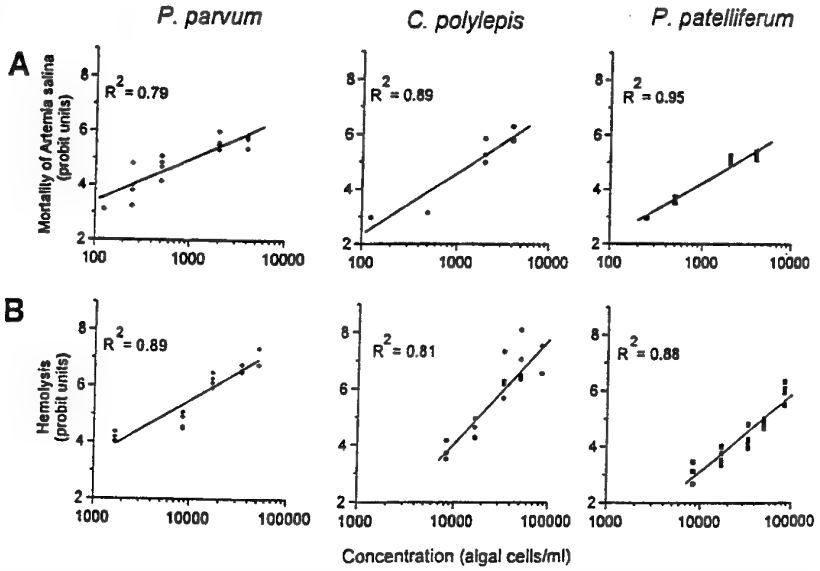

FIGURE 1. Dose-response relationship for the effects of fresh culture and extract of *Prymnesium parvum, Chrysochromulina polylepis,* and *P. patelliferum* on different test systems. The amount of algal extract is given as the corresponding number of cells extracted. (A) Mortality of *Artemia salina* by fresh algae. (B) Hemolytic activity by crude algal extract.

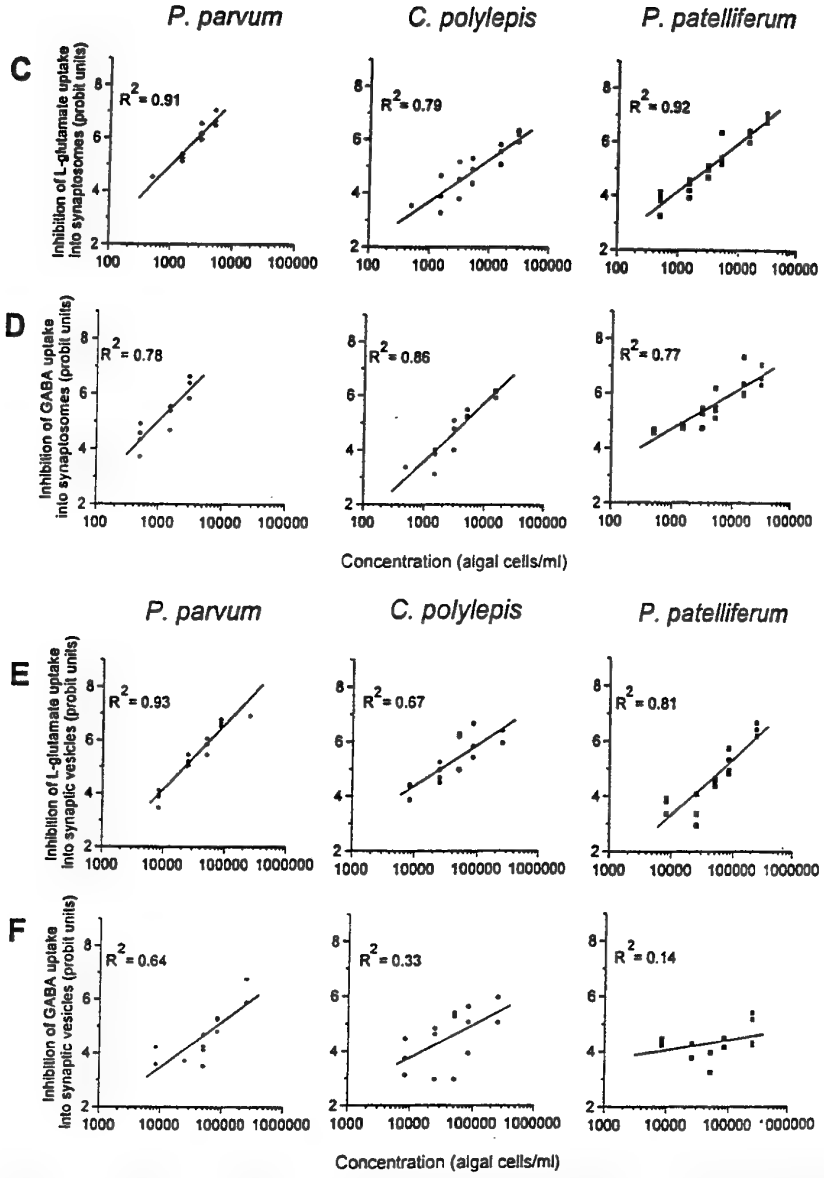

FIGURE 1 (*Continued*). (C) Inhibition of uptake of L-glutamate into synaptosomes by crude algal extract. (D) Inhibition of uptake of γ-aminobutyric acid (GABA) into synaptosomes by crude algal extract. (E) Inhibition of uptake of L-glutamate into synaptic vesicles by crude algal extract. (F) Inhibition of uptake of γ-aminobutyric acid (GABA) into synaptic vesicles by crude algal extract.

TABLE 1. Average Cell Concentration of *Prymnesium parvum*, *Chrysochromulina leadbeateri*, *P. patelliferum*, and C. *leadbeateri* Causing 50% Mortality of the Brine Shrimp *Artemia salina* (LC50), 50% of Total Hemolysis (EC50), and 50% Inhibition of Glutamate or GABA into Synaptosomes or Synaptic Vesicles (IC50)

Algal species	Algal culture, mortality of *A. salina*, LC50 (cell ml^{-1})	Algal extract						
		Hemolysis EC50 (cell ml^{-1})	Inhibition of synaptosomal uptake			Inhibition of vesicular uptake		
			IC50 (cells ml^{-1})		IC50 ratio, Glu : GABA	IC50 (cells ml^{-1})		IC50 ratio, Glu : GABA
			Glutamate	GABA		Glutamate	GABA	
P. parvum	1100	5600	1000	1000	1 : 1	22,000	92,000	1 : 4
C. polylepis	1600	18,000	6900	4700	1 : 0.7	26,000	114,000	1 : 4
P. patelliferum	2400	49,000	2900	1800	1 : 0.6	69,000	>250,000	—
C. leadbeateri	>100,000	>800,000	>143,000	>143,000	—	>250,000	>250,000	—

Note. The values were obtained from the regression lines of the dose-response relationships shown in Figure 1. Probit value 5 represents 50% effect. The amount of algal extract is given as the equivalent number of cells extracted.

Crude algal extracts were tested for toxic activity using three different test methods: the hemolysis test, the synaptosome test, and the vesicle test. The ranking in toxicity of the algal cultures obtained in the *Artemia* bioassay (*P. parvum* > *C. polylepis* > *P. patelliferum*) was found also in the hemolysis test and in the vesicle test (Table 1). The synaptosomal neurotransmitter uptake, however, was less sensitive to the *C. polylepis* extract than to the extracts of the two *Prymnesium* species. Comparing the two *Prymnesium* species, the ratios between the LC50 values for *P. patelliferum* and *P. parvum* in the *Artemia* test and between the respective IC50 values in the synaptosome test and the vesicle test were 2–3. The difference in hemolytic activity, however, was more marked, and the EC50 ratio between the two species, respectively, was about 9. In accordance with the nontoxicity to *A. salina* by the cultures of *C. leadbeateri*, no effects of the lipid extract of *C. leadbeateri* were shown on synaptosomes or synaptic vesicles at extract concentrations corresponding to 143,000 and 250,000 cells ml^{-1}, respectively. Low hemolytic activity (about 20% hemolysis) was observed with extract corresponding to 800,000 cells ml^{-1} (results not shown), a concentration that far exceeded 100% hemolysis for the extracts of the 3 other species.

The slopes of the lines showing the dose-response relationship of the four different test methods were not markedly different for the three species (Figure 1). In the hemolysis test the line obtained with *C. polylepis* was slightly steeper than those obtained with *P. parvum* or *P. patelliferum* (Figure 1B), whereas for the inhibition of neurotransmitter uptake into synaptosomes and synaptic vesicles the steepest lines were obtained with extract of *P. parvum* (Figure 1, C–E).

The relative inhibition of the uptake of L-glutamate and GABA into synaptosomes or synaptic vesicles, determined as the IC50 ratio between the uptake systems for L-glutamate and GABA, were similar with all the toxic extracts (Table 1). In synaptosomes, this ratio was 1 : 1, 1 : 0.7, and 1 : 0.6 with the extracts of *P. parvum*, *C. polylepis*, and *P. patelliferum*, respectively. In synaptic vesicles, the situation was different and a ratio between the IC50 values of the uptake of L-glutamate and GABA of about 1 : 4 was obtained with extract of both *P. parvum* and *C. polylepis*. The effect of the *P. patelliferum* extract on synaptic vesicles was low, and IC50 of the GABA uptake could not be determined for extract up to a concentration corresponding to 250,000 cells ml^{-1}.

The sensitivity of the synaptosome test was similar to the sensitivity of the *Artemia* bioassay. The hemolysis test showed lower sensitivity followed by the vesicle test.

DISCUSSION

In the present study we have used four different test methods to compare the toxic activities of axenic cultures of the marine phytoflagellates *P. parvum*, *P. patelliferum*, *C. polylepis*, and *C. leadbeateri* isolated from

ichthyotoxic algal blooms. The cultures of *P. parvum*, *P. patelliferum*, and *C. polylepis* were lethal to the test organism *A. salina*, and the crude lipid extracts of these algal cultures showed toxic effects toward human erythrocytes and toward synaptosomes and synaptic vesicles of rat brain. In contrast, cultures of *C. leadbeateri* were nontoxic to *A. salina*, and the crude lipid extracts of the algal cultures showed no toxic activity. The negative results of the tests with cultures and lipid extracts of *C. leadbeateri* indicated that the effects on red blood cells, synaptosomes, and synaptic vesicles by extracts of *P. parvum*, *P. patelliferum*, and *C. polylepis* were not caused by general algal metabolites, but most probably caused by lipophilic toxic compounds that also killed the *A. salina* nauplii.

Prymnesium parvum and *P. patelliferum* are nearly identical morphologically (Green et al., 1982), but showed a clear difference in toxicity as determined by all four test methods. The difference in hemolytic activity was more marked compared to the difference in activity shown with the three other test methods. These results indicate that, when grown under identical conditions, compounds with hemolytic activity are produced in proportionally higher amounts by *P. parvum* than by *P. patelliferum*.

Compared to the *Prymnesium* extracts, the extract of *C. polylepis* was not as potent to the synaptosomal neurotransmitter uptake as could have been expected from its hemolytic activity and inhibitory effect on the vesicular neurotransmitter uptake (Table 1). This result agrees with our earlier studies on lipid extracts of *P. patelliferum* and *C. polylepis*, where the highest inhibitory activity toward synaptosomal neurotransmitter uptake was shown in extracts of *P. patelliferum* (Meldahl et al., 1993). The effect on synaptosomes by the algal extracts thus appeared to be caused by toxin(s) that were slightly different from those affecting the red blood cells and the synaptic vesicles.

The ratios between the IC50 values of the synaptosomal uptake of L-glutamate and GABA were similar for extracts of *P. parvum*, *C. polylepis*, and *P. patelliferum* (Table 1). This indicates a similar mechanism of action on the synaptosomal membrane by the lipid extracts of the three toxic species. The precise mechanism of action on the synaptosomal membrane by the toxic substances in these algae has not been elucidated. For the crude lipid extract of *P. patelliferum*, we found no inhibition of the ATPase activity in the membranes of synaptosomes (unpublished results), and we have suggested that the inhibition of the uptake systems in synaptosomes by crude extract of *P. patelliferum* may be due to a membrane depolarization (Meldahl et al., 1994).

The vesicular uptake of L-glutamate was more sensitive to inhibition by the algal extracts than the uptake of GABA. An IC50 ratio between the two uptake systems of 1 : 4 was obtained with extract of both *P. parvum* and *C. polylepis*. The extract of *P. patelliferum* showed only low activity in this test, but the results point toward a similar difference in inhibition of the two uptake systems. In agreement with the conclusion from the synaptosome

test, the similar selective inhibition of the two uptake systems in synaptic vesicles suggests that toxin(s) of the three algal extracts may act in a similar way on the vesicles. Since it is believed that L-glutamate uptake into synaptic vesicles is more dependent on the membrane potential than is the GABA uptake (Maycox et al., 1988; Hell et al., 1990), it is possible that the stronger effect on the L-glutamate uptake by algal toxins was caused by a membrane depolarization.

There was a difference in sensitivity among the methods used to test the toxicity of the algal extracts. The synaptosome test was most sensitive, followed by the hemolysis test and the vesicle test. This could partly be due to the differences in amount of tissue material present in the test mixture, which has been shown to be of importance in the synaptosome test (unpublished results), and partly to the difference in the composition of the membranes that were affected. Martin and Padilla (1971) suggested that cholesterol and lecithin in the erythrocyte membrane were the attaching sites of the toxin molecules and that lysis occurred after a prelytic period while the absorption of toxin on the erythrocytes took place. Using liposomes, it has been shown that the amount of *P. parvum* toxin required for lysis increased with decreasing content of cholesterol in the membrane (Imai & Inoue, 1974). The cholesterol concentration in the membrane of both synaptosomes and synaptic vesicles is about half of that of the red blood cells (Dodge & Phillips, 1967; Breckenridge et al., 1972, 1973). The only significant difference between the synaptosomal membrane and the synaptic vesicles in lipid composition is that the synaptosomal membrane is rich in gangliosides, whereas synaptic vesicles are almost devoid of these glycolipids. The latter property may be involved in the higher sensitivity of the synaptosomes compared to the synaptic vesicles.

We believe that the three different test preparations used in the present work will be useful in the study of the mechanism of action on biological membranes exerted by the toxins of these ichthyotoxic marine flagellates.

In conclusion, our results showed that toxicity of each of the related flagellates *P. parvum*, *P. patelliferum*, and *C. polylepis*, grown under similar conditions, could be demonstrated by all the four test methods used in this study. Since the relative degree of toxicity of one algal species compared to the others differed slightly depending on the test method used, it may be that each species produces slightly different mixtures of similar toxic structures.

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PAPER III

Meldahl, A.-S. and Fonnum, F (1995) The effects of a purified toxic extract of *Prymnesium patelliferum* on transport of ions through the plasma membrane of synaptosomes. *Toxicon*, **33**, 1071-1086.

THE EFFECTS OF A PURIFIED TOXIC EXTRACT OF *PRYMNESIUM PATELLIFERUM* ON TRANSPORT OF IONS THROUGH THE PLASMA MEMBRANE OF SYNAPTOSOMES

by

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ABSTRACT

MELDAHL, A.-S. and FONNUM, F. The effects of a purified toxic extract of *Prymnesium patelliferum* on transport of ions trough the plasma membrane of synaptosomes. *Toxicon* ••, ••• - •••, 19••. — Extract of the ichthyotoxic marine alga *Prymnesium patelliferum* has been shown to have several different effects on the transport of neurotransmitters across nerve membranes. It inhibits the sodium dependent uptake of L-glutamate and GABA and enhances the calcium dependent release of acetylcholine. We have therefore investigated the effects of a purified toxic algal extract on some membrane properties using rat brain synaptosomes. We found that under conditions where the algal extract inhibited the uptake of L-glutamate, it increased the intracellular concentrations of Na^+ and Ca^{2+}, stimulated efflux of K^+ determined as ^{86}Rb efflux, and depolarised the synaptosomal membrane. There was no effect on Na^+/K^+-ATPase or ouabain-insensitive ATPase activities. Further there was no leakage of the cytosolic marker LDH indicating that the various effects of the algal extract were not due to nonspecific leakage or lysis of the synaptosomes. The rise in the cytosolic concentration of free Ca^{2+} induced by the algal extract was dependent on extracellular Ca^{2+}, and was inhibited by flunarizine (1-100 μM) but not by the Ca^{2+} channel blockers ω-conotoxin GVIA (1 μM), diltiazem (100 μM), nifedipine (100 μM) or verapamil (100-500 μM). The increase in Na^+-influx induced by the algal extract was insensitive to tetrodotoxin (3 μM) and procaine (100 μM) whereas both the Na^+-influx and the membrane depolarisation were inhibited by flunarizine (1-100 μM). The increase in K^+-efflux was insensitive to flunarizine (5-100 μM). From these results it appears that the toxic extract of *P. patelliferum* increases the permeability of synaptosomes to Ca^{2+}, Na^+ and K^+ and that these effects may be responsible for the plasma membrane depolarization and the disturbance of the neurotransmitter transport processes.

INTRODUCTION

The lipid extract of the ichthyotoxic marine alga *Prymnesium patelliferum* has been shown to have several effects on the transport of neurotransmitters across nerve membranes. The algal extract is a potent inhibitor of the high affinity uptake of L-glutamate and γ-aminobutyric acid (GABA) into isolated nerve terminals (synaptosomes) of rat brain (MELDAHL and FONNUM, 1993). Further, at higher concentrations, it inhibits the uptake of L-glutamate, GABA and dopamine into synaptic vesicles and causes lysis of human erythrocytes (MELDAHL and FONNUM, 1993). It also induced release of acetylcholine from a smooth muscle preparation (MELDAHL *et al.*, unpublished). Due to the high sensitivity, the method for determination of high affinity uptake of L-glutamate into synaptosomes has formed the basis of a method to detect the presence of toxins in natural water samples during algal blooms of this and related potentially ichthyotoxic algae (MELDAHL and FONNUM 1991; MELDAHL *et al.*, 1994a). It is not known whether the various effects of the extract of *P. patelliferum* are caused by one or more toxic compunds. The chemical structure of the toxin(s) of *P. patelliferum* is not characterized, but may be similar to the structure of the toxins of the morphologically closely related species *P. parvum*, which exerts effects similar to those of *P. patelliferum* (MELDAHL *et al.*, 1994a). Two hemolytically active digalactomonoglycerols with highly unsaturated acyl chains (KOZAKAI *et al*, 1982) as well as two partly defined polyhydroxy-polyene-polyether structures (IGARASHI *et al.*, 1993) have been isolated from *P. parvum*. The latter compounds were 1,000 times more hemolytically active than commercial saponin, lethal to both fish (*Oryzias latipes*) and mice, and induced influx of Ca^{2+} into rat glioma C6 cells.

The mechanism of action underlying the various toxic effects of the extract of *P. patelliferum* has not previously been investigated. Since L-glutamate uptake into synaptosomes constitute a sensitive method for detecting the toxic activity, we have investigated the effect of the algal extract on some properties of neurotransmitter transport in synaptosomes. The uptake of L-glutamate across the plasma membrane is highly dependent on the inwardly directed Na^+-gradient and the outwardly directed K^+-gradient, both maintained by the Na^+/K^+-ATPase

(KANNER and SCHULDINER, 1987). Moreover, the transport is stimulated by a negative membrane potential (NICHOLLS and ATTWELL, 1990). The release of neurotransmitters is highly dependent on an increase in the Ca^{2+}-concentration of the nerve terminal. Interference with either of the factors involved in the regulation of the neurotransmitter transport systems would be expected to cause a decrease in the accumulation of neurotransmitters. Here we report the effects of a purified toxic exract of *P. patelliferum* on Na^+/K^+-ATPase, efflux of K^+, membrane potential, and the concentration of intracellular free sodium ($[Na^+]_i$) and intracellular free calcium ($[Ca^{2+}]_i$) in synaptosomes of rat brain. The effects on these parameters are discussed in relation to the inhibitory effect on the synaptosomal high affinity uptake of L-[^{3}H]glutamate.

Some preliminary results have previously been reported (MELDAHL *et al.*, 1994b).

MATERIALS AND METHODS

Chemicals

Pluronic F-127 and the fluorescent indicators sodium-binding benzofuran isophthalate-acetoxymethyl ester (SBFI/AM), fura-2-acetoxymethyl ester (fura-2/AM) and di-1,1', 3,3,3',3'-hexamethylindodicarbocyanine iodide ($DiIC_1(5)$) were purchased from Molecular probes Inc., Oregon, USA. Anhydrous dimethylsulfoxide (DMSO) was from Aldrich-Chemie, Steinheim, Germany. L-[^{3}H]glutamate and ^{86}rubidium chloride was from New England Nuclear, Boston, Mass., USA. Diltiazem hydrochloride, flunarizine dihydrochloride, gramicidin, ionomycin, procaine, tetrodotoxin, tetraethyl ammonium chloride, verapamil hydrochloride and bovine serum albumin (Fraction V) were purchased from Sigma Chemical Company, St. Louis, MO, USA. ω-Conotoxin GVIA was from Peninsula Laboratories Inc., Belmont, CA, USA, and nifedipine from Research Biochemicals Inc., Mass., USA. All other chemicals were of analytical laboratory reagent grade. Due to light sensitivity, nifedipine was dissolved in ethanol immediately before use and protected from exposure to light.

Preparation of algal extract

The axenic strain of *Prymnesium patelliferum* (strain N) originated from a sample collected from a toxic algal bloom in Hylsfjorden, south-western Norway, 3 August 1989, and was grown as previously described (MELDAHL *et al.*, 1994a). Crude toxic extract was obtained by extraction as described by ULITZUR and SHILO (1970). One volume of the concentrated algal culture was mixed with four volumes of methanol - chloroform (1:2), and the lower chloroform fraction was evaporated to dryness in a rotary evaporator. The dry extract was dissolved in chloroform and purified further by the method of KALUZNY *et al.* (1985) using an aminopropyl bonded phase column (Analytichem Bond Elut, 100 mg, Varian, Harbour City, CA, USA). The aminopropyl column was washed under vacuum (5-10 kPa) with two 1 ml portions of hexane. The crude algal extract dissolved in chloroform was applied to the column and allowed to run through under vacuum. Then the column was washed with 2 ml of chloroform - 2-propanol (2:1) and 2 ml of diethyl ether - acetic acid (98:2). The toxic fraction was eluted with methanol, dried under N_2, dissolved in methanol, and stored in darkness at -20°C without loss of activity until testing. The biological activity of the eluted fractions was determined by a hemolysis assay (MELDAHL *et al.*, 1994a) and an assay for determination of high affinity uptake of L-[^{3}H]glutamate into synaptosomes (see below). Activity was detected only in the fraction eluted with methanol. Thin layer chromatography of the active fraction on precoated silica plates (Merck) eluted with chloroform - methanol - H_2O (75:25:4), and sprayed with 2% blood cell suspension in 0.13 M NaCl, pH 5.5, showed only two or three hemolytically active spots (R_f values: 0.06, 0.33, 0.41) instead of the six spots (R_f values: 0.06, 0.15, 0.23, 0.33, 0.41, 0.79) emerging from the original crude extract. The concentration of algal extract is given as the original number of algal cells determined in an electronic particle counter (Coulter Electronics Ltd., model D).

Preparation of synaptosomes

Synaptosomes were prepared from brains of male Wistar rats (200-250 g, Møllegaard Breeding Laboratories, Denmark) as described previously by GRAY and WHITTAKER (1962).

Brains were homogenized in 0.32 M sucrose (5% wt/vol). The homogenate was centrifuged at 1,000 × *g* for 10 min, and the supernatant was further centrifuged at 13,000 x *g* for 30 min. The pellet (P_2) was resuspended in 0.32 M sucrose and layered onto a discontinuous sucrose gradient consisting of 1.2 M and 0.8 M sucrose and centrifuged in a swing-out rotor (Contron TST 28.38) at 65,000 × *g* for 30 min. The synaptosomal fraction was isolated from the band between 1.2 M and 0.8 M sucrose and washed by resuspension in three volumes of 0.32 M sucrose and centrifugation at 13,000 × *g* for 20 min. The pellet was resuspended in the appropriate medium as indicated.

Determination of uptake of L-[^{3}H]glutamate

High affinity uptake of L-[^{3}H]glutamate into synaptosomes was determined by the method of FONNUM *et al.* (1980). Briefly, synaptosomes (protein concentration 0.1 mg × ml^{-1}) were preincubated with the algal extracts at 25 °C for 15 minutes in Tris buffered medium consisting of 15.0 mM Tris-HCl (pH 7.4), 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1.2 mM Na_2HPO_4, 10 mM glucose, bubbled with O_2 for 15 minutes. Uptake of L-[^{3}H]glutamate (97 nM, 17.30 Ci × $mmol^{-1}$) was conducted for 1 min at 25 °C and was terminated by filtration and washing with 0.15 M NaCl containing bovine serum albumin (0.5 mg × ml^{-1}). Controls contained the same concentrations of methanol as the samples with algal extracts (0.2-0.6%), and methanol showed no effect on the basal uptake of L-[^{3}H]glutamate. Each assay was carried out in triplicate. The inhibition by the algal extracts was expressed as per cent of control after subtraction of values of blank samples (synaptosomes in 0.32 M sucrose, incubated at 0°C) which represented about 10% of the total uptake. Where indicated the synaptosomes were preincubated with flunarizine for 10 min.

Determination of Na^+/K^+-ATPase activity

The Na^+/K^+-ATPase activity was determined in synaptosomal plasma membranes (SPMs) as described in principle by NAGAFUJI *et al.* (1992). The synaptosomal plasma membranes were obtained by exposing the pelleted synaptosomes to a hypotonic shock in a solution of 0.1 mM EGTA and 10 mM Tris-HCl (pH 7.4). The suspension was centrifuged at 13,000 × g for

30 min and the resulting pellet was resuspended in the storage medium containing 0.32 mM sucrose, 4 mM $MgCl_2$ and 0.4 mM EDTA. The preparation was stored in liquid N_2 without loss of ATPase activity until testing within 3 weeks. The Na^+/K^+-ATPase activity of the SPMs was determined in a reaction mixture containing SPMs (protein concentration 0.1 mg × ml^{-1}) in 10 mM Tris-HCl (pH 7.4), 130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$ and 0.4 mM EGTA with or without 0.25 mM ouabain (final volume 0.5 ml). Reactions were started by addition of 3 mM Tris buffered ATP (vanadate-free, pH 7.4). After incubation at 30 °C for 15 min, the reaction was terminated by addition of 125 µl of ice cold 6 % (vol/vol) perchloric acid followed by incubation on ice for 10 min. The samples were centrifuged at 15,000 × *g* for 5 min. The liberated inorganic phosphate (P_i) was determined by the phosphomolybdate method as described by MEDZIHRADSKY *et al.* (1971). One volume of freshly prepared solution consisting of 144 mM $FeSO_4$, 8.15 mM ammonium molybdate and 0.58 M H_2SO_4 was added to one volume of the supernatant. After 5 min incubation at room temperature, the absorbance at 700 nm was determined (Shimadzu UV-1201). Standards in the linear range of 10-50 nmol of P_i (K_2HPO_4) were included. The Na^+/K^+-ATPase activity was obtained by subtracting the ouabain-insensitive basal ATPase activity from total ATPase activity.

Determination of intracellular sodium concentration, $[Na^+]_i$

The intracellular sodium concentration, $[Na^+]_i$, was measured with the Na^+-binding fluorescent probe SBFI/AM as described by DERI and ADAM-VIZI (1993). SBFI acts in a manner similar to fura-2 (MINTA and TSIEN, 1989) and an increase in the fluorescence excitation ratio (I_{340}/I_{380}) indicates an increase in the intracellular concentration of Na^+. The synaptosomes were resuspended in 0.32 M sucrose (20 mg × ml^{-1}) and kept on ice until use. The dye was dissolved in anhydrous DMSO. Immediately before administration, SBFI/AM solution was mixed with an equal volume of Pluronic F-127 [20% (wt/vol)] in DMSO. The synaptosomes (2 ml, protein concentration 3 mg × ml^{-1}) were loaded with SBFI by incubation in the presence of 10 µM SBFI/AM at 30 °C for 60 min in the standard medium containing 25 mM HEPES (4-(2-hydroxyethyl) -1-piperazineethanesulfonic acid) (pH 7.4), 128 mM NaCl, 2.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, bubbled

with O_2 for 15 min. At the end of the incubation period, the synaptosomes were sedimented by centrifugation at 13,000 × g for 15 min and the pellet was washed with the standard incubation medium, resuspended in the medium (protein concentration 1.2 mg × ml^{-1}), and kept on ice. Aliquots of 100 µl were added to 1100 µl of prewarmed (30 °C) medium (final protein concentration 0.1 mg × ml^{-1}). Determination of SBFI-mediated fluorescence was performed on a computerised Perkin-Elmer LS50 luminescence spectrometer, excitation wavelength changing between 340 and 380 nm (10 nm slit) and emission wavelength 509 nm (10 nm slit). A water jacketed cuvette holder maintained the sample at a constant temperature of 30 °C, and the sample was stirred continuously with a magnetic stirrer. Where indicated the synaptosomes were preincubated with inhibitors of Na^+ influx for 10 min.

Determination of membrane potential

Membrane potential was measured using the potential-sensitive fluorescent probe $DiIC_1(5)$ as described by EHRENGRUBER *et al.* (1993). Stock solutions of $DiIC_1(5)$ were made up in ethanol-H_2O (1:9) to 2.0 mM and stored at -20 °C. Synaptosomes were resuspended to a protein concentration of 2.4 mg × ml^{-1} in standard incubation medium used for the measurement of $[Na^+]_i$. The synaptosomal suspension was kept on ice during the experiment. Aliquots of synaptosomes were added to the incubation medium prewarmed to 30 °C and containing 2 M $DiIC_1(5)$. Determinations of $DiIC_1(5)$-mediated fluorescence in synaptosomes were performed on a computerised Perkin-Elmer LS50 luminescence spectrometer, excitation wavelength 620 nm (10 nm slit) and emission wavelength 665 (15 nm slit). A water jacketed cuvette holder maintained the sample at a constant temperature of 30 °C and the sample was stirred continuously with a magnetic stirrer. Where indicated the synaptosomes were preincubated with flunarizine for 10 min.

Determination of intracellular Ca^{2+} concentration, $[Ca^+]_i$

Intracellular free calcium $[Ca^+]_i$ was measured using the fluorescent probe fura-2/AM as described by YATES *et al.* (1992). Fura-2/AM was dissolved in DMSO immediately before use. Synaptosomes were resuspended in Ca^{2+}-free standard medium (protein concentration 10 mg

$\times$ ml^{-1}) with 5 µM fura-2/AM and incubated for 40 min at 30 °C. At the end of the incubation period, the synaptosomes were sedimented by centrifugation at 13,000 x g for 15 min and the pellet resuspended in the Ca^{2+}-free standard incubation medium (protein concentration 1.2 mg x ml $^{-1}$), and kept on ice. The fluorescence measurements with fura-2 were performed in the same manner as with SBFI described above. Choline was substituted for Na^+ in the low Na^+-medium. Where indicated the synaptosomes were preincubated with inhibitors of Ca^{2+} influx for 10 min.

Determination of $^{86}Rb^+$-efflux

The determination of $^{86}Rb^+$-efflux from synaptosomes was modified from the method of BARTSCHAT and BLAUSTEIN (1985). The synaptosomes were resuspended in the standard incubation medium without Ca^{2+} and containing 0.1 mM RbCl and $^{86}Rb^+$ (~ 30 µCi $\times$ ml^{-1}). The protein concentration was about 5 mg $\times$ ml^{-1}. The suspension was incubated for 30 min at 30 °C to load the synaptosomes with $^{86}Rb^+$, and then kept on ice until used in the experiment. The efflux of $^{86}Rb^+$ was determined by incubation of the $^{86}Rb^+$-loaded synaptosomes in standard incubation medium containing 1.2 mM $CaCl_2$ and 0.1 mM RbCl (0.5 ml, final protein concentration 0.1 mg $\times$ ml^{-1}) with or without the extract of *P. patelliferum* at 30 °C for various time periods (10 sec - 20 min). The $^{86}Rb^+$-efflux was terminated by the rapid addition of 2 ml of stopping solution (also 30 °C), filtration (Millipore HAWP, diam. 25 mm, pore size 0.45 m) and washing with 2 ml stopping solution. The stopping solution contained: 145 mM tetraethyl ammonium chloride, 5 mM RbCl, 5 mM $MgCl_2$, 10 mM $NiCl_2$, 20 mM HEPES (pH 7.4). The radioactivity remaining on the filter was determined by liquid scintillation spectroscopy. Each assay was carried out in duplicate. The $^{86}Rb^+$-efflux in the presence of methanol (0.2-0.6%) was not different from control incubations. The relative $^{86}Rb^+$-efflux is presented as per cent radioactivity remaining on the filter compared to control after subtraction of the nonspecific filter binding (10-20 %) determined by addition of 0.3 % Triton X-100.

Determination of lactate dehydrogenase (LDH) leakage

The method for determination of leakage of cytosolic LDH from synaptosomes was modified from the method of WILSON and KIRSHNER (1983). Synaptosomes were suspended (protein concentration 0.1 mg × ml^{-1}) in HEPES-buffer containing: 20 mM HEPES (pH7.4), 123 mM NaCl, 5.0 mM KCl, 2.0 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 5.6 mM glucose, bubbled with oxygen for 15 minutes. The suspension was incubated with the algal extract at 37 °C for 15 min. The incubation was terminated by centrifugation at 15,000 × *g* for 3 min. An aliquot (100 µl) of the supernatant was mixed with 3 ml reaction substrate containing 20 mM Tris-HCl (pH 7.4), 113 µM β-NADH and 330 µM sodium pyruvate. The rate of oxidation of NADH was recorded automatically for 3 minutes with 15 seconds intervals at 340 nm (Beckman DU-50 Series Spectrophotometer). The degree of LDH leakage was determined from the slope of the absorbance curve. Maximum leakage of LDH was obtained with 0.3% Triton X-100.

Determination of protein concentration

Protein concentration was measured as described by LOWRY *et al.* (1951) using bovine serum albumin as standard.

RESULTS

Effect on the uptake of L-[^{3}H]glutamate

The concentration-dependent inhibition of the uptake of L-[^{3}H]glutamate after 15 min pretreatment with the extract of *P. patelliferum* is shown in Fig. 1. The concentration of algal extract giving 50 % inhibition of the uptake of L-[^{3}H]glutamate (IC_{50}) was determined to be 22,500 cells × ml^{-1}. Almost total inhibition was obtained at a concentration of algal extract corresponding to 200,000 cells × ml^{-1}. The degree of inhibition was not dependent upon the presence of Ca^{2+} (data not shown). In the presence of 1 µM or 5 µM flunarizine, the inhibitory effect of the algal extract on the L-[^{3}H]glutamate uptake was increased. At a concentration of algal extract of 10,000 cells × ml^{-1} the inhibitory effect was increased from 40% to 91% or

96%, respectively (data not shown). Flunarizine alone did not affect the uptake of L-[^{3}H]glutamate at 1 μM but at 5 μM the uptake was inhibited by 65 % (data not shown)

Effect on Na^+/K^+-ATPase activity

The Na^+/K^+-ATPase activity of the synaptosomal membrane is required for the maintenance of the electrochemical Na^+-gradient which provides the driving force for the uptake of glutamate. The enzyme activity, determined as production of inorganic phosphate, was measured in synaptosomal plasma membranes obtained by lysis of the synaptosomes in a hypotonic solution. Incubation of the synaptosomal plasma membranes (protein concentration 0.1 mg × ml^{-1}) with the algal extract for 15 min resulted in only a slight inhibition of the enzyme activity (Table 1). The treatment with extract of 10^6 algal cells × ml^{-1} reduced the enzyme activity by only 16%, indicating that the synaptosomal Na^+/K^+-ATPase activity was not affected to an extent that could explain the inhibition of the uptake of L-[^{3}H]glutamate caused by the extract of *P. patelliferum*. The ouabain-insensitive ATPase activity was not significantly changed by the treatment with algal extract (Table 1).

Effect on $[Na^+]_i$

The possibility that the algal extract interfered with the transmembrane Na^+-concentration gradient in synaptosomes was directly examined using the fluorescent probe, sodium binding benzofuran isophthalate acetoxymethyl ester, SBFI/AM (MINTA and TSIEN, 1989). Figure 2A shows that addition of the extract of *P. patelliferum* caused an increase in the fluorescence ratio which reached a stable level within 1-2 min. The effect of the algal extract was compared with maximum increase in Na^+ obtained with the ionophore gramicidin at 5 μM. Fifty percent increase in $[Na^+]_i$ was obtained by extract of approximately 10,000 cells × ml^{-1}. Hence, the algal extract was about twice as effective in increasing $[Na^+]_i$ as in inhibiting the uptake of L-glutamate (Fig. 1). Methanol had no effect on the fluorescence intensity ratio and the algal extract did not interfere with the fluorescence properties of SBFI. To investigate whether toxin present in the algal extract interfered with endogenous Na^+-channels of the nerve terminal, the synaptosomes were treated with blockers of the voltage-dependent sodium channel,

tetrodotoxin (3 μM) and the local anestethic procaine (100 μM), each binding to different sites on the channel (PAUWELS *et al.*, 1986). Neither compound was able to alter the effect of the algal extract at 100,000 cells × ml^{-1} (data not shown). Pretreatment with the diphenylmethylpiperizine flunarizine (1-10 μM), recently reported to block both voltage-dependent Na^+-channels and voltage-dependent Ca^{2+}-channels in cerebrocortical synaptosomes of rat brain (COUSIN *et al.*, 1993), caused a marked concentration dependent attenuation of the Na^+-influx induced by the algal extract at 100,000 cells × ml^{-1} (Fig. 2B).

Effect on the membrane potential

The potential sensitive dye $DiIC_1(5)$ was used to investigate the possible effect of the extract of *P. patelliferum* on the membrane potential. Addition of algal extract at a concentration of 100,000 cells × ml^{-1} to the synaptosomal suspension (protein concentration 0.1 mg × ml^{-1}) containing the dye, resulted in an increase in the fluorescence intensity indicating a decrease in membrane potential (Fig. 3A, inset). A stable intensity level was obtained within 1 - 2 minutes. Methanol, the extract vehicle (1 % vol/vol) had no significant effect on the membrane potential and the algal extract did not interfere with the fluorescence properties of the dye (data not shown). Figure 3A shows the concentration-dependent effect of the algal extract relative to total depolarization by 5 μM gramicidin. The concentration of toxic extract giving 50% depolarization was determined from the curve to be about 75,000 cells × ml^{-1}. This concentration exceeded the 50% effect-concentration for the inhibition of the uptake of L-[^{3}H]glutamate (Fig. 1) and for the increase in $[Na^+]_i$ (Fig. 2A). Pretreatment of the synaptosomes with flunarizine (1-100 μM), a compound reported to inhibit influx of both Na^+ and Ca^{2+} in synaptosomes (COUSIN *et al.*, 1993), inhibited the membrane depolarisation caused by the algal toxin (Fig. 3B). Flunarizine alone did not interfere with the fluorescence signal of $DiIC_1(5)$.

Effect on $[Ca^{2+}]_i$

Based on the marked effects of the extract of *P. patelliferum* on the Na^+-gradient and the membrane potential of synaptosomes, it was of interest to investigate the effects on the influx

of Ca^{2+}. The intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) was measured with the membrane permeable Ca^{2+} binding probe, fura-2/AM. Addition of algal extract to synaptosomes (protein concentration 0.1 mg × ml^{-1}) loaded with fura-2 caused a rise in the fluorescence intensity ratio (I_{340}/I_{380}) indicating an increase in $[Ca^{2+}]_i$. About half maximum increase in intracellular Ca^{2+}, as compared with the effect of the Ca^{2+}-ionophore ionomycin at 2 μM, was obtained with extract of 100,000 cells × ml^{-1} (Fig. 4A, upper trace). A stable level in fluorescence ratio was reached within 1 minute. This increase in the intracellular Ca^{2+}-concentration could be due either to influx of extracellular Ca^{2+} and/or induction of release of Ca^{2+} from the intracellular Ca^{2+}-storage organelles. However, after chelation of the extracellular Ca^{2+} with 2 mM EGTA, addition of algal extract showed no increase in the fluorescence ratio (Fig. 4A), showing that release of Ca^{2+} from intracellular stores was probably not involved in the increase in $[Ca^{2+}]_i$ induced by the toxin. The induction of Ca^{2+}-influx by the algal toxin was not altered by substitution of Ch^+ for Na^+ (data not shown) or by the pretreatment with tetrodotoxin (3 μM) to block the voltage-dependent Na^+-channels (data not shown). The calcium channel antagonists ω-conotoxin (1-5 μM), verapamil (100 -500 μM), diltiazem (100 μM) and nifedipine (100 μM) were all ineffective in blocking the influx of Ca^{2+} induced by the algal extract (data not shown). However, flunarizine (1-100 μM) markedly suppressed the increase in Ca^{2+} influx induced by the algal extract at 100,000 cells × ml^{-1} (Fig. 4B).

Effect on $^{86}Rb^+$-efflux

The effect of the algal extract on the K^+-gradient of synaptosomes was determined by measuring the efflux of loaded $^{86}Rb^+$. As previously shown by BARTSCHAT and BLAUSTEIN (1985), synaptosomes transport K^+ and Rb^+ in a similar fashion. Moreover, $^{86}Rb^+$ is a more convenient isotope to use than $^{42}K^+$ due to the short half-life of $^{42}K^+$. The algal toxin caused a dose-dependent increase in efflux of $^{86}Rb^+$ (Table 2). The increase was detected after 10 sec and reached a maximum within 1-3 min (data not shown). Omission of Ca^{2+}, or lowering the Na^+-concentration by substitution with Ch^+ did not alter the effect of the algal toxin on the efflux of $^{86}Rb^+$ (data not shown). To examine whether flunarizine was able to inhibit this

effect, the synaptosomes incubated in the standard incubation medium were pre-treated with 5, 10 and 100 μM flunarizine for 10 min before addition of the algal extract. Flunarizine had no effect on the degree of $^{86}Rb^+$ efflux induced by the algal extract (data not shown).

Leakage of lactate dehydrogenase

There was no increase in the activity of the cytosolic marker lactate dehydrogenase in the incubation medium after 15 min treatment of the synaptosomes (protein concentration 0.1 mg × ml^{-1}) with the extract of *P. patelliferum* at concentrations up to 8 x10^{-6} cells × ml^{-1} (data not shown).

DISCUSSION

In the present study we have investigated the effects of a purified toxic extract of *P. patelliferum* on several membrane functions of rat brain synaptosomes. Synaptosomes were selected because these are particles with several well established membrane properties that can be assayed. We have previously found that the inhibition of L-glutamate uptake by the algal extract was dependent on the amount of synaptosomal protein (MELDAHL *et al.*, 1994c). Therefore, in order to compare the sensitivity of the various processes required to drive the neurotransmitter uptake, the experiments designed to measure the effect on gradients of K^+, Na^+ and Ca^{2+}, the membrane potential, the Na^+/K^+-ATPase activity and the leakage of cytosolic LDH, were carried out at a constant protein concentration. The results showed that under conditions in which the algal extract inhibited the high affinity uptake of L-[^{3}H]glutamate into synaptosomes (Fig. 1), it increased the intracellular concentrations of Na^+ (Fig. 2A) and Ca^{2+} (Fig. 4A), increased the efflux of K^+ (Table 2) and depolarized the synaptosomal membrane (Fig. 3A). There was no effect on the Na^+/K^+-ATPase activity or on the efflux of the cytosolic marker LDH, the latter indicating that the effects described were not likely due to lysis of the synaptosomes, or formation of large holes in the membrane.

The importance of the electrochemical gradient of Na^+ as a driving force for the high affinity uptake system for neurotransmitters is well established (KANNER and SHARON, 1978; BREW and ATTWELL, 1987; KANNER and SCHULDINER, 1987; NICHOLLS and ATTWELL, 1990). Thus, the transport is sensitive to ionophores such as monensin, gramicidin D and nigericin which collapse the Na^+-gradient (KANNER and SHARON, 1978; ROSKOSKI *et al.*, 1981) and to ouabain which inhibits the Na^+/K^+-ATPase (STALLCUP *et al.*, 1979). Moreover, the neurotoxin veratridine which opens the voltage dependent Na^+-channel, inhibits transport of L-glutamate and GABA, and tetrodotoxin, which blocks the Na^+-channel, reverses this inhibition (KANNER, 1980). Transport is enhanced by valinomycin which hyperpolarizes the membrane (ROSKOSKI *et al.*, 1981). The results of the present study show that at the concentration required to give 50% inhibition of the L-glutamate uptake (22,500 cells $\times$ ml^{-1}) (Fig. 1) there was no significant effect on the Na^+/K^+-ATPase or the K^+-gradient, about 20% membrane depolarization (Fig. 3A), and more than 50% increase in the intracellular Na^+-concentration (Fig. 2A). Thus, both the membrane depolarization and the reduction in the Na^+-concentration gradient may be responsible for the observed inhibition of the uptake of L-glutamate caused by the extract of *P. patelliferum*. It should be noted, however, that since this algal extract is only partly purified this correlation between the effects of the various parameters measured may not be entirely valid. If several different toxins are present in the extract it is possible that each of them may have different profiles of activity.

The increase in Na^+-permeability was insensitive to inhibition by TTX (3 μM) and procaine (100 μM), both blockers of the voltage-dependent Na^+-channels acting at different sites in the channel molecule, indicating little or no involvement of voltage-dependent Na^+-channels.

The algal extract increased the intracellular free [Ca^{2+}]. This effect was dependent on extracellular Ca^{2+} since after removal of extracellular Ca^{2+} by addition of EGTA there was no effect of the algal extract (Fig. 4A). These results are consistent with the results of a preliminary study by IGARASHI *et al.* (1993) showing that a toxin of *P. parvum* induced influx of Ca^{2+} into rat glioma C6 cells. Although no further details were given in this communication, the similar effect of toxins of *P. parvum* and *P. patelliferum* on the membrane-flux of Ca^{2+} supports the assumption of a close relationship between toxins present in these two species

(MELDAHL *et al.*, 1994a) which are morphologically closely related (GREEN *et al.*, 1982). Various hypotheses could be put forward for the mechanism involved in the Ca^{2+}-influx induced by the algal extract. First, it could trigger Ca^{2+}-influx directly through voltage dependent Na^{+}-channels or through voltage dependent Ca^{2+}-channels. These possibilities seem unlikely based on the fact that the increase in intracellular $[Ca^{2+}]_i$ was not altered by treatment with tetrodotoxin (data not shown) or by the Ca^{2+}-channel blockers ω-conotoxin (1-10 μM, verapamil (100-500 μM), nifedipine (100 μM) and diltiazem (100 μM) (data not shown). The latter results should, however, be interpreted with caution since previous studies have shown that voltage-sensitive Ca^{2+}-channels of rat brain synaptosomes show low sensitivity to dihydropyridine Ca^{2+}-antagonists and only weak sensitivity to ω-conotoxin GVIA (NACHSHEN and BLAUSTEIN, 1979; DANIELL *et al.*, 1983; CARVALHO *et al.*, 1989; SUSZKIW *et al.*, 1989; KOBAYASHI *et al.*, 1992). This probably reflects that the synaptosomes lose some factor(s) essential for coupling the binding of blockers to the physiological effects (CARVALHO *et al.*, 1988). Another possible route of Ca^{2+} entry, is via the membrane Ca^{2+}/Na^{+} exchanger, a mechanism that normally extrudes Ca^{2+} but that might operate in reverse direction under conditions with elevated cytosolic $[Na^{+}]$ (NACHSHEN *et al.*, 1986). This possibility seems less likely since substituting choline for sodium did not alter the increase in $[Ca^{2+}]_i$ by addition of the algal extract (Fig. 4B).

Flunarizine, a diphenylmethylpiperizine, appeared to be a potent inhibitor of the increase in the intracellular $[Na^{+}]$ and $[Ca^{2+}]$ as well as the membrane depolarisation exerted by the extract of *P. patelliferum*. Flunarizine is widely acknowledged as a Ca^{2+}-channel blocker in a number of tissues (TODD and BENFIELD, 1989) and has also been found to inhibit Na^{+}-channels (PAUWELS *et al.*, 1991; COUSIN *et al.*, 1993). Inhibition of K^{+}-induced increase in $[Ca^{2+}]_i$ by flunarizine in synaptosomes has previously been demonstrated (WIBO and GODFRAIND, 1983; CARVALHO *et al.*, 1989; KOBAYASHI *et al.*, 1992; COUSIN *et al.*, 1993) as well as inhibition of veratridine- and ouabain-induced Na^{+}-influx (COUSIN *et al.*, 1993). Flunarizine has been shown to be an effective protective agent against veratridine-induced cell damage in neuronal cell cultures (PAUWELS *et al.*, 1989, 1990, 1991; RICH and HOLLOWELL, 1990) and hippocampal slices (ASHTON *et al.*, 1990). The mechanism of action of flunarizine remains

speculative. Unlike many Ca^{2+} antagonists, flunarizine is very hydrophobic, even in its charged state (< pH 7.7) (THOMAS and SEELIG, 1993). VOGELSANG *et al.* (1985) have suggested that flunarizine may displace Ca^{2+} associated with negatively charged phospholipids of the plasma membrane and thereby protect the membrane from the lipid reorganization occurring for example during brain ischemia. The finding that the increase in $^{86}Rb^{+}$ efflux was insensitive to inhibition by flunarizine indicate that the compound is not generally protecting the synaptosome membrane against all effects of the toxin of *P. patelliferum*. Moreover, flunarizine was not able to suppress the inhibitory action of the algal extract on the uptake of L-glutamate. In contrast, the presence of a low concentration of flunarizine (1 μM) resulted in a potentiation of the inhibitory effect of the algal extract. At a higher concentration (5 μM) flunarizine alone appeared to be a potent inhibitor of the synaptosomal uptake of L-glutamate, probably due to its inhibitory action on the transport of Na^{+}. These effects of flunarizine will be the subject for a separate study.

Altogether, the extract of *P. patelliferum* apparently increases the permeability to Na^{+}, Ca^{2+} and K^{+} in the synaptosomal membrane, along with membrane depolarization and inhibition of the net uptake of L-glutamate. The 50% effect concentrations of the algal extract on each parameter increased in the order: Na^{+}-influx < L-glutamate uptake < membrane depolarization ≈ Ca^{2+}-influx < K^{+}-efflux. The marked decrease in the Na^{+}-gradient was probably responsible for the inhibition of L-glutamate uptake, via a reversal of the L-glutamate carrier. Moreover, the decrease in the Na^{+}-gradient may also be involved in the membrane depolarization. The increase in Ca^{2+}-influx could be a result of the membrane depolarization, and it can not be excluded that a part of the Ca^{2+}-influx in synaptosomes induced by the extract of *P. patelliferum* was occurring through voltage-activated Ca^{2+}-channels since most known Ca^{2+}-channel blockers show low potency towards the voltage-activated Ca^{2+}-channels of synaptosomes. This would agree with the increase in voltage-activated Ca^{2+}-currents of cultured pituitary cells caused by this algal extract (MELDAHL *et al.*, 1994b). Moreover, inhibition of phosphatase activity by the extract of *P. patelliferum* has been detected in synaptosomes (A.- S. MELDAHL, unpublished observations). Hence, the increase in Ca^{2+}-influx could be connected to an opening of voltage-dependent Ca^{2+}-channels via modulation of

channel proteins by phosphorylation. Alternatively, based on the amphiphilic character of the toxin structures isolated from *P. parvum* (KOZAKAI *et al.*, 1982; IGARASHI *et al.*, 1993), which are probably similar to the toxins present in *P. patelliferum*, it is possible that the toxin(s) of the *P. patelliferum* extract, by perturbing the membrane bilayer in some way, is able to form pores allowing the increase in the permeability to ions such as Na^+, Ca^{2+} and K^+.

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TABLE 1. THE EFFECT OF THE TOXIC EXTRACT OF *P. PATELLIFERUM* ON THE ACTIVITY OF Na^+/K^+-ATPASE AND OUABAIN-INSENSITIVE ATPASES OF SYNAPTOSOMAL PLASMA MEMBRANES§

Concentration of algal extract (algal cells x ml^{-1})	**Na^+/K^+ATPase (% of control)**	**Ouabain-insensitive ATPases (% of control)**
Control	100	100
10,000	94 ± 3	95 ± 4
100,000	88 ± 4	99 ± 6
1,000,000	84 ± 2 **	88 ± 12

§Synaptosomal plasma membranes (protein concentration 0.1 mg × ml^{-1}) were incubated with the extract of *P. patelliferum* (15 min, 30 °C) in Tris-buffer in the presence or absence of 0.25 mM ouabain. The Na^+/K^+-ATPase activity was obtained by subtracting the ouabain-insensitive activity from the total activity. The results are presented as percent of control in each experiment and represent the mean ± S.E.M. of three or four experiments in duplicate. The control enzyme activity was 303 ± 32 nmol P_i × 0.1 mg $protein^{-1}$ × ml^{-1} for the Na^+/K^+ ATPase activity and 228 ± 26 nmol P_i × 0.1 mg $protein^{-1}$ × ml^{-1} for the ouabain-insensitive ATPases. Test for significance of difference from control was performed using Students *t*-test. $^{**}P < 0.01$.

TABLE 2. THE EFFECT OF THE EXTRACT OF *P. PATELLIFERUM* ON EFFLUX OF $^{86}Rb^+$ FROM SYNAPTOSOMES§

Concentration of algal extract (algal cells x ml^{-1})	$^{86}Rb^+$ remaining on the filter (% of control)
60,000	81 ± 13
200,000	59 ± 17
600,000	33 ± 19 *

§Synaptosomes (protein concentration 0.1 mg × ml^{-1}) were incubated at 30 °C for 3 min in the presence of the algal extract in normal medium containing 0.1 mM RbCl. The results are presented as per cent $^{86}Rb^+$ remaining on the filter compared to control in each experiment. The values represent the mean ± S.E.M. of three experiments in duplicate. Test for significance of difference from control was performed using Students *t*-test. *$P < 0.05$.

LEGENDS FOR FIGURES

FIGURE 1. THE EFFECT OF THE EXTRACT OF *PRYMNESIUM PATELLIFERUM* ON THE UPTAKE OF L-[^{3}H]GLUTAMATE INTO SYNAPTOSOMES OF RAT BRAIN.

The synaptosomes (protein concentration 0.1 mg × ml^{-1}) were incubated with the algal extract for 15 min at 25 °C in Krebs-Tris-buffer. The uptake of L-[^{3}H]glutamate was terminated after 1 min by filtration and subsequent washings with 0.13 M NaCl with BSA (0.5 g × ml^{-1}). The results are expressed as percent of control in each experiment and represent the mean ± S.E.M. of seven or eight experiments in triplicate.

FIGURE 2. THE EFFECT OF THE EXTRACT OF *P. PATELLIFERUM* ON THE INTRACELLULAR CONCENTRATION OF FREE Na^+ IN SYNAPTOSOMES.

The intracellular concentration of free Na^+ was determined as the ratio between the fluorescence excitation at 340 nm and 380 nm (emission at 509 nm) of the Na^+ binding probe SBFI. Aliquots of synaptosomes loaded with SBFI/AM were incubated in 1.2 ml of prewarmed (30 °C) HEPES-buffered medium to give a protein concentration of 0.1 mg × ml^{-1}. Extract of *P. patelliferum* was added as indicated. The traces shown are representative for two or three independent experiments. **(A)** The effect of the algal extract on fluorescence ratio of synaptosomes in standard incubation medium. **(B)** The effect of the algal extract (100,000 cells × ml^{-1}) on intracellular [Na^+] after 10 min pretreatment with different concentrations of flunarizine.

LEGENDS FOR FIGURES (cont.)

FIGURE 3. THE EFFECT OF THE EXTRACT OF *P. PATELLIFERUM* ON THE MEMBRANE POTENTIAL OF SYNAPTOSOMES DETERMINED USING THE POTENTIAL SENSITIVE PROBE $DiIC_1(5)$. Aliquots (50 µl) of synaptosomes were added to 1,150 µl of prevarmed (30 °C) HEPES-buffered medium to give a protein concentration of 0.1 mg × ml^{-1}. The traces shown are representative for two to four independent experiments. **(A)** After generation of a stable membrane potential, different concentrations of algal extract was added. The membrane depolarization induced by the algal extract is presented as percentage of the maximum depolarization obtained with 5 M gramicidin. Each point represents the mean ± S.E.M. of 3 to 6 measurements. Inset: A trace showing the time-course of the membrane depolarization by the addition of the algal extract at a concentration of 100,000 cells × ml^{-1} **(B)** The effect of the algal extract (500,000 cells × ml^{-1}) on the membrane potential after 10 min pretreatment with different concentrations of flunarizine.

FIGURE 4. THE EFFECT OF THE EXTRACT OF *P. PATELLIFERUM* ON THE INTRACELLULAR CONCENTRATION OF FREE CA^{2+} IN SYNAPTOSOMES.

The intracellular concentration of free Ca^{2+} was determined as the ratio between the fluorescence excitation at 340 nm and 380 nm, (emission at 509 nm) of the Ca^{2+} binding probe fura-2. Aliquots of synaptosomes (100 M) loaded with fura-2/AM were added to 1,100 l of prewarmed (30 °C) HEPES-buffered medium without Ca^{2+} to give a protein concentration of 0.1 mg × ml^{-1}. $CaCl_2$ (2 mM) or EGTA (2 mM) and extract of *P. patelliferum* (100,000 cells × ml^{-1}) was added as indicated. The traces shown are representative for two or three independent measurements. **(A)** The effect of the algal extract (100,000 cells × ml^{-1}) on the intracellular concentration of Ca^{2+} in the presence (upper trace) and the absence (lower trace) of Ca^{2+}. **(B)** The effect of the algal extract (100,000 cells × ml^{-1}) on the intracellular concentration of Ca^{2+} after 10 min pretreatment with different concentrations of flunarizine.

Figure 1

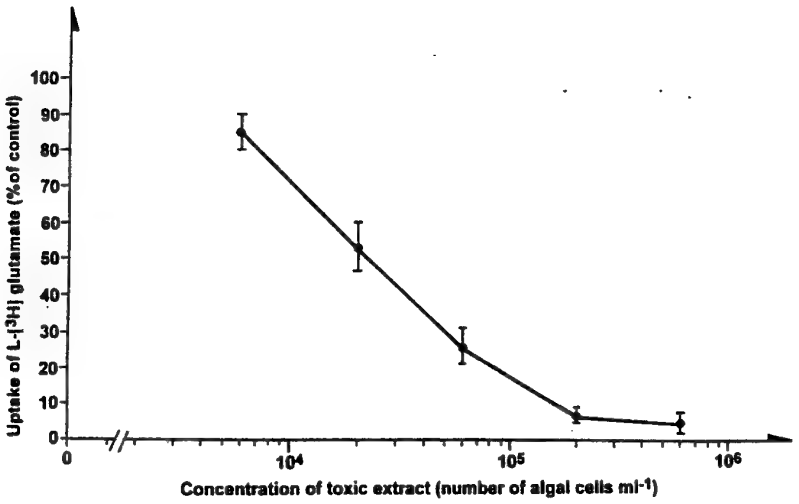
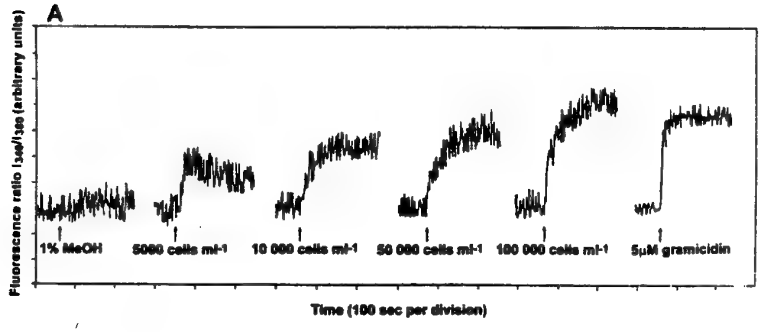


Figure 2



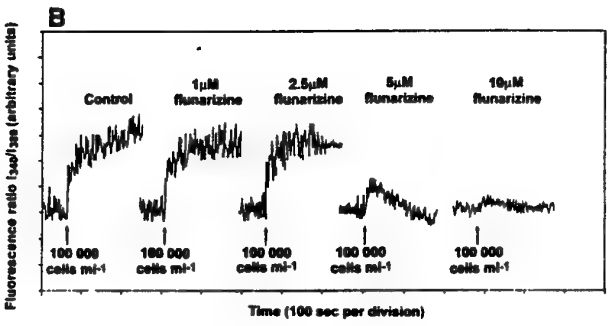
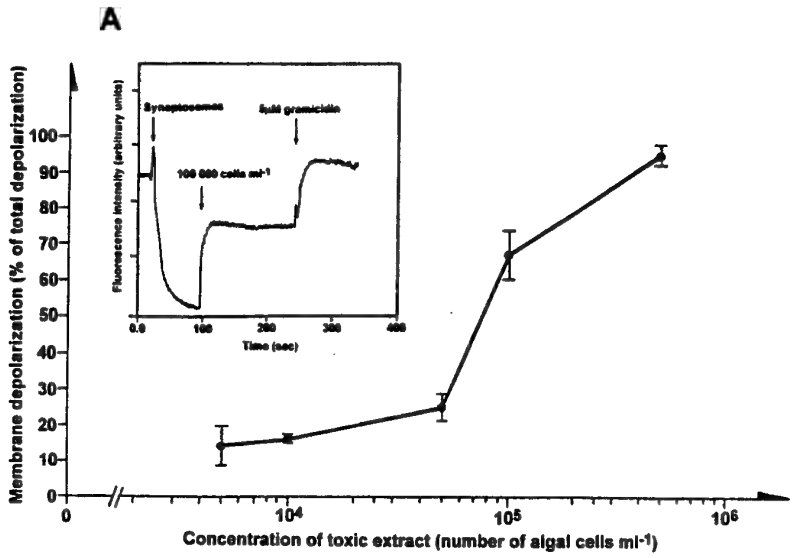


Figure 3


A
Membrane depolarization (% of total depolarization)
Concentration of toxic extract (number of algal cells ml-1)
Fluorescence intensity (arbitrary units)
Time (sec)
Synaptosomes
fluid gramicidin
100 000 cells ml-1

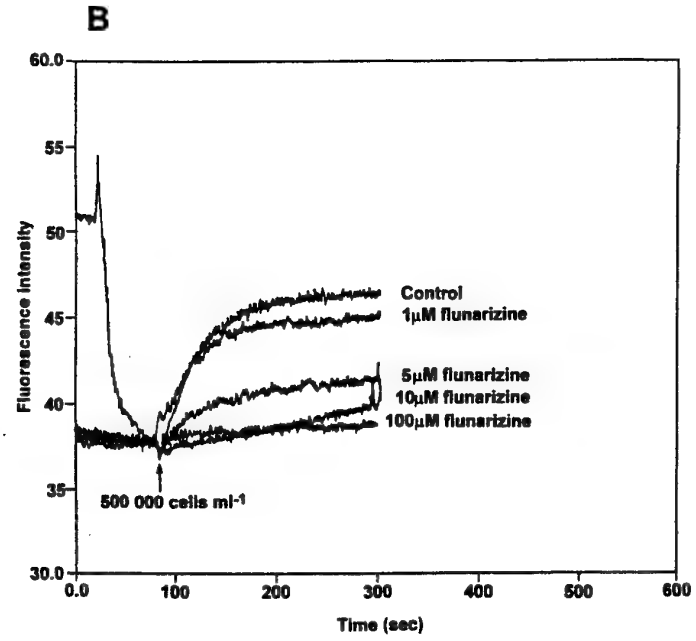
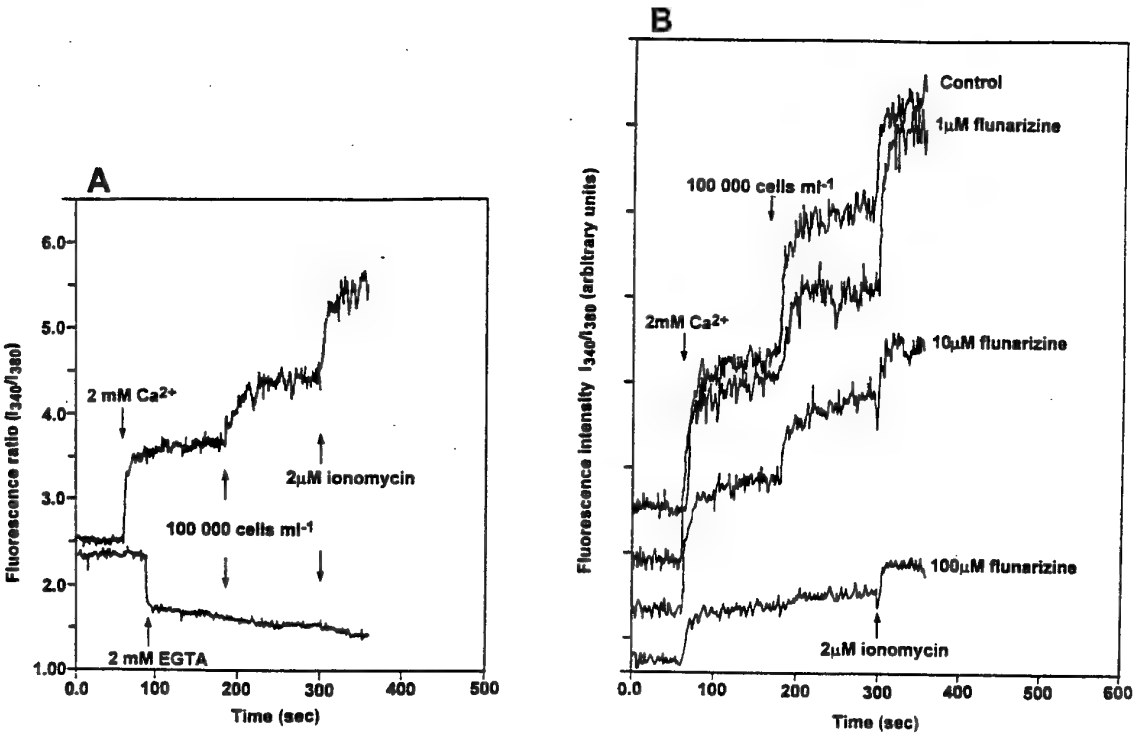

B
Fluorescence intensity
Time (sec)
Control
1µM flunarizine
5µM flunarizine
10µM flunarizine
100µM flunarizine
500 000 cells ml-1

Figure 4


A
Fluorescence ratio (I_{340}/I_{380})
6.0
5.0
4.0
3.0
2.0
1.00
2 mM Ca^{2+}
2μM ionomycin
100 000 cells ml^{-1}
2 mM EGTA
0.0
100
200
300
400
500
Time (sec)
B
Fluorescence intensity I_{340}/I_{380} (arbitrary units)
Control
1μM flunarizine
100 000 cells ml^{-1}
2mM Ca^{2+}
10μM flunarizine
100μM flunarizine
2μM ionomycin
0.0
100
200
300
400
500
600
Time (sec)

PAPER IV

Kolderup, J., Meldahl, A.-S., Eriksen, S., Haug, E., Fonnum, F. and Sand, O (1995). Toxin of the marine alga *Prymnesium patelliferum* enhances voltage dependent Ca^{2+}-currents, elevates the cytosolic Ca^{2+} -concentration and facilitates hormone release in clonal rat pituitary cells. *Acta Physiol. Scand*, **154**, 321-327.

Acta Physiol Scand 1995, **000**, 000-000

Toxin of the marine alga *Prymnesium patelliferum* enhances voltage dependent Ca^{2+}-currents, elevates the cytosolic Ca^{2+}-concentration and facilitates hormone release in clonal rat pituitary cells

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Short title: Effects of *Prymnesium* toxin on pituitary cells

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The marine flagellate *Prymnesium patelliferum* produces toxins lethal to fish. The toxin extracted from the alga has haemolytic, cytotoxic and neurotoxic effects, but the action mechanisms of the toxin is not known in detail. We have examined the toxin effects on the voltage sensitive Ca^{2+}-currents, the cytosolic Ca^{2+}-level ($[Ca^{2+}]_i$) and the prolactin release in clonal rat anterior pituitary GH_4C_1 cells, which possess T- and L-type Ca^{2+}-channels. The trans-membrane Ca^{2+}-current was recorded using whole-cell voltage clamp. After 5-15 minutes exposure to the algal toxin at a final concentration of 50 000-100 000 cells ml^{-1}, the Ca^{2+}-currents through both the T- and L-channels showed a 2-3 fold enhancement. The voltage sensitivity of the Ca^{2+}-currents was not affected by the algal toxin, and the toxin-induced currents were inhibited by 100 μM of the Ca^{2+}-channel blocker D-600. In toxin-exposed cells microfluorometric measurements based on fura-2 revealed an increase of $[Ca^{2+}]_i$ from 100-150 nM to 300-500 nM. This elevation was delayed and partially inhibited by 100 μM D-600. The algal toxin induced prolactin release in a dose-dependent manner, and this effect was inhibited by the Ca^{2+}-channel blocker verapamil. We therefore conclude that the the toxin of *P. patelliferum* affects the Ca^{2+} homeostasis of the pituitary cells by increasing the leak through voltage sensitive Ca^{2+}-channels, resulting in increased $[Ca^{2+}]_i$ and secretion of prolactin.

Keywords: Ca^{2+}, Ca^{2+}-channels, fura-2, lactotrophs, pituitary cells, prolactin, *Prymnesium patelliferum*, voltage-clamp

Seasonal blooming of the ichthyotoxic marine flagellates *Prymnesium patelliferum* and *P. parvum* is a major problem for fish-farming in the brackish water of the fjords of south-western Norway, where such blooms have killed 150-750 metric tons of farmed salmonid fish per year (Eikrem & Throndsen, 1993). Toxin extracted from *P. parvum* has cytotoxic effects on a variety of biological test systems, causing cell-lysis at high concentrations and after prolonged exposure (see Paster, 1973).

Prymnesium patelliferum is closely related to *P. parvum*, and extracts of the two species exert similar effects, such as inhibition of neurotransmitter transport into synaptosomes and synaptic vesicles from rat brain and haemolysis of human red blood cells (Meldahl *et al.,* 1994a). The toxic compunds of *P. patelliferum* have not been characterized, but may be similar to those of *P. parvum*. Two haemolytically active digalactomonoglycerols with highly unsaturated acyl chains have been isolated from *P. parvum* (Kozakai *et al*., 1987). In a preliminary communication Igarashi *et al*. (1995) have reported two partly defined polyhydroxy-polyene-polyether structures. The latter compounds were 1 000 times more haemolytically active than commercial saponin, lethal to both fish (*Oryzias latipes*) and mice, and induced influx of Ca^{2+} in rat glioma C6 cells.

We have recently found that the toxin extracted from *P. patelliferum* depolarizes the plasma membrane and increases the permeability to Na^{+}, Ca^{2+} and K^{+} in synaptosomes from rat brain (Meldahl & Fonnum, unpublished). In the present study we have used the prolactin producing clonal rat anterior pituitary GH_4C_1 cells, which possess T- and L-type Ca^{2+}-channels, as model system to examine the effects of the algal toxin on voltage dependent Ca^{2+}-channels. We report that the extract of *P. patelliferum* enhances the currents through both T- and L-type Ca^{2+}-channels, leading to increased intracellular concentration of Ca^{2+} and enhanced prolactin release. Some preliminary results of this study have previously been reported (Meldahl *et al*. 1994b).

MATERIALS AND METHODS

Drugs. Verapamil and its analog D-600 were purchased from Knoll AG, Ludwigshafen, Germany, and tetrodotoxin (TTX) was obtained from Sigma Chemical Company, St. Louis, MO, USA.

Preparation of algal extract. The axenic strain of *Prymnesium patelliferum* (strain N) was isolated from a sample collected from a toxic algal bloom in Hylsfjorden, south-western Norway, 3 August 1989, and was grown as previously described (Meldahl *et al.*, 1994a). Crude toxic extract of the algae was obtained by extraction as described by Ulitzur & Shilo (1970). One volume of the concentrated algal culture was mixed with 4 volumes of methanol-chloroform (1:2), and the lower chloroform fraction was evaporated to dryness in a rotavapor. The dry extract was dissolved in methanol and stored at -20 °C. The amount of algal extract is given as the number of cells from which the extract was obtained. The cell number was determined in an electronic particle counter (Coulter Electronics Ltd., model D).

Cell-culture. The original establishment of the GH strains of rat pituitary tumor cells was performed by Tashjian *et al.* (1968). These cells spontaneously synthesize and secrete prolactin and/or growth hormone. The GH_4C_1 subclone used in the present study mainly produces prolactin. The cells were grown as monolayer cultures in plastic tissue culture flasks containing Ham's F-10 medium supplemented with 6% (v/v) horse serum and 2.5% (v/v) foetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every 3-4 days. The cells used for the recordings were seeded in 35 mm plastic Petri dishes 3-6 days before the experiments took place. Prior to an experiment the culture medium was replaced with Hepes buffered salt solution (HBSS) of the following composition (mM): NaCl 150, KCl 5, $MgCl_2$ 1.3, $CaCl_2$ 2, glucose 10 and Hepes (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulphonic acid) 10, buffered to pH 7.4 with NaOH.

Electrophysiology. The measurements were carried out as described by Sand *et al.* (1989), using the patch-clamp method in the whole-cell configuration (Hammil

et al., 1981). The fire-polished patch electrodes had a resistance of 4-10 MΩ. For the initial recordings of the resting membrane potential and input resistance, the patch electrodes were filled with an internal solution of the following composition (mM): K-gluconate 140, KCl 5, $MgCl_2$ 1, Mg-ATP 2, EGTA (ethylene glycol-bis(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid) 0.5, Hepes/NaOH 5, pH 7.2. For recordings of Ca^{2+}-currents, the potassium salts of the internal solution were replaced with the corresponding caesium salts to eliminate current through the K^+-channels, and 1 µM TTX was added to the external solution to block current through the Na^+-channels. To facilitate Ca^{2+}-currents, the external Ca^{2+}-concentration was raised to 10 mM, with a corresponding reduction of the NaCl-concentration. The electrodes were connected to a List L/M-EPC7 patch clamp amplifier and standard recording equipment. The recorded membrane potential was adjusted for the junction potential between the intracellular solution and the bath solution. The cells were viewed through an inverted microscope during the experiments, which were conducted at room temperature.

Microfluorometry of fura-2 loaded cells. For the microfluorometric measurements the cells were seeded in dishes with a glass coverslip sealing a central, 15 mm whole in the bottom. The cells were loaded with the fluorescent Ca^{2+}-indicator fura-2 by exposure to 2 µM fura-2/AM (Molecular Probes Inc., USA) in HBSS for 30-60 min at 37 ^oC. After washout of fura-2/AM in the extracellular milieu, the cells were further incubated for 30 min at room temperature. The dish was then mounted on an Olympus inverted microsocope, forming the central part of the Olympus OSP-3 system, for dual excitation microfluorometry. The excitation side of this system consists of a xenon-lamp connected to a rotating mirror unit fitted with appropriate interference filters. The excitation light was in this way switched at 200 Hz between 340 nm and 380 nm. The emitted fluorescence was measured at 500 nm by means of a third interference filter and a photomultiplier. The measurements were restricted to single cells using a pinhole diaphragm, and the absolute Ca^{2+}-concentration was estimated from the ratio between the emissions at the excitation

wavelengths of 340 nm and 380 nm (Grynkiewicz *et al.,* 1985). Calibration was obtained by recording the fluorescence ratio between 340 nm and 380 nm excitition light for droplets of 20 mM Pipes-NaOH buffer (pH 6.8) containing 10 μM fura-2 and different ratios of Ca-EGTA/EGTA, chosen to give concentrations of free Ca^{2+} covering the physiological, cytosolic range (Harafuji & Ogawa, 1980).

Measurement of prolactin release. The secretion of stored prolactin was measured in culture dishes as the amount of hormone accumulating in the HBSS during a 15 min incubation period at 37 °C. Prior to the incubation the cells were washed three times to remove extracellular prolactin. The incubation was terminated by placing the tubes on ice, followed by centrifugation (500 g, 4 min, 4 °C). The supernatant was stored at -20 °C until measurement of prolactin using a radioimmunoassay specific for rat prolactin (Haug & Gautvik, 1976). When indicated, the cells were pretreated for 5 min with the Ca^{2+}-channel blocker verapamil before exposure to the algal extract. The experiments were conducted in triplicate.

RESULTS

Effects of P. patelliferum *toxin on the resting membrane potential and input resistance*

The effects of the toxin extract on the resting membrane potential and input resistance of the GH_4C_1 cells were studied using the whole-cell patch clamp method in current clamp mode. Addition of the extract of *P. patelliferum* at a final concentration of 50 000-100 000 cells ml^{-1} to the recording solution did not acutely disturb the resting membrane potential or resistance. In both the controls and the treated cells these passive membrane properties were within the normal range of -40--60 mV and 2-4 G Ω, respectively. However, after 30-60 min dramatic changes in these parameters were

observed, probably due to cell lysis. The membrane potential was reduced to 0--10 mV and the input resistance dropped to less than 100 MΩ. At higher toxin concentrations (i.e. 200 000 cells ml^{-1}) these destructive changes of the membrane properties were acute, and became evident within the first minute of toxin exposure. The toxin effects on these membrane parameters were not investigated further in the present study.

Effects of P. patelliferum *toxin on the voltage sensitive* Ca^{2+}*-currents*

The effects of the algal extract on the trans-membrane Ca^{2+}-currents were examined using the whole-cell patch clamp method in voltage clamp mode. The toxic extract was applied at a final concentration of 50 000-100 000 cells ml^{-1}, and the recordings were performed before the deterioration of the passive membrane properties The holding potential was -67 mV and the 300 ms command potentials were altered in 10 mV steps between -87 mV and +43 mM. The time between each step was 1 s.

Fig. 1a shows a typical current trace recorded from a cell depolarized to -7 mV in normal solution containing 1 μM TTX. The Cs^{+} in the filling solution precludes outward K^{+}-currents, and the external TTX excludes inward Na^{+}-current. The evoked inward current is thus carried by Ca^{2+}. The current has evidently both a transient and a more persistent component, corresponding to currents through T- and L-type Ca^{2+}-channels. We did not employ different holding potentials or specific channel blockers to isolate the two current components. A crude separation was instead achieved by measuring the initial peak of the inward current and the sustained current 250 ms after onset of the command potential.

After 5-15 min exposure to algal extract and until lysis both the transient (T-current) and the sustained (L-current) current component increased 2-3 times compared to controls (Fig. 1b). Methanol, the extract vehicle, had no detectable effect on the inward current (data not shown).

The magnitude of the inward current showed some variation between the different cell culture batches. It was therefore necessary to normalize the current measurements to be able to compare the data obtained throughout the experimental period of several months. This was achieved by presenting all the current measurements obtained on a particular day, from every cell and at the different command potentials, as a percentage of the mean, transient current peak recorded at -7 mV from all the control cells on that day.

The current/voltage relations for the transient and sustained current components presented in Fig. 1c and d includes normalized data from 67 control cells and 57 toxin exposed cells recorded within the time-window described above. Toxin exposure enhanced the maximum transient current component from 100±9 % (SEM) to 259±34 %, and the maximum sustained current component from 32±4 % to 66±9 %. However, the voltage dependence of the inward current components was not affected by the algal extract. The two inward current components in both the control cells and the toxin exposed cells were effectively blocked by 100 μM of the Ca^{2+}-channel blocker D-600 (Fig. 1c, d).

Effects of P. patelliferum *toxin on* $[Ca^{2+}]_i$

It is reasonable to assume that enhancement of the inward Ca^{2+}-current, as described above, might cause an elevation of $[Ca^{2+}]_i$. This was confirmed by microfluorometric measurements from GH_4C_1 cells loaded with fura-2. The effect of exposure to the algal extract at a final concentration of 100 000 cells ml^{-1} was recorded either by following a single cell for 30-40 min (Fig. 2), or by recording from a different cell in the dish every 50 s during a similar time period. The effects of toxin exposure on $[Ca^{2+}]_i$ was recorded in 5 dishes, and in all cases $[Ca^{2+}]_i$ increased from 100-150 nM to 300-500 nM after 5-20 min exposure to the algal extract. In 3 additional dishes the toxin extract was applied together with 100 μM D600, and in these cases the elevation of $[Ca^{2+}]_i$ was delayed and partially inhibited. However, the

presence of D-600 did not prevent or delay the toxin-induced lysis of the cells after 30-60 min (data not shown).

Effects of P. patelliferum *toxin on prolactin secretion*

Changes of $[Ca^{2+}]_i$ is one of the key factors in the control of prolactin release from pituitary cells. The toxin-induced elevation of $[Ca^{2+}]_i$ might therefore be expected to facilitate hormone release from the GH_4C_1 cells. This assumption was confirmed by incubating culture dishes with different concentrations of the algal extract for 15 min, and measuring the prolactin accumulated in the medium. The toxin of *P. patelliferum* increased the secretion of prolactin in a dose-dependent manner (Fig. 3). Moreover, in the presence of 100 μM of the Ca^{2+}-channel blocker verapamil, this stimulation of prolactin release was inhibited.

Treatment with high concentration of algal extract ($1x10^6$ cells ml^{-1}) caused massive efflux of prolactin, indicating cell lysis (data not shown). The cells then formed pseudopodia-like extrusions, but did not stain with trypan blue.

DISCUSSION

The present investigation shows that the toxin present in the extract of the marine alga *P. patelliferum* induces secretion of prolactin from the clonal GH_4C_1 cells. This effect is apparently a consequence of the increased $[Ca^{2+}]_i$ due to enhanced influx of Ca^{2+} through voltage sensitive Ca^{2+}-channels, since organic blockers of these channels inhibited both the elevation of $[Ca^{2+}]_i$ and the increased hormone release. This is in accordance with the well recognized relationship between elevation of $[Ca^{2+}]_i$ and hormone secretion in pituitary cells (see Ozawa & Sand, 1986).

The voltage sensitivity of the Ca^{2+}-currents was not affected by the algal toxin, and the current enhancement caused by the algal extract was inhibited by 100

μM of the Ca^{2+}-channel blocker D-600. It is highly unlikely that the toxin forms new types of Ca^{2+}-conducting channels with exactly the same voltage dependency as the endogenous Ca^{2+}-channels, and with a similar sensitivity to D-600. Our findings therefore indicate that the enhanced inward Ca^{2+}-currents were due to increased influx through the normal, endogenous Ca^{2+}-channels. This may either be caused by elevated current carrying capacity of the individual channels, or by increased number of active channels in the membrane. The latter possibility is feasible, since a significant fraction of the Ca^{2+}-channels in the membrane under normal, physiological conditions may be in an inactivated, unphosphorylated state (Armstrong & Eckert, 1987). Since the algal toxin influenced the currents through the T- and L-type of Ca^{2+}-channels to a similar degree, it probably interacts with structures common for both these channel types.

At a concentration of 100 000 cells ml^{-1}, the effects of the algal toxin on Ca^{2+}-currents and $[Ca^{2+}]_i$ in the GH_4C_1 cells occured after a delay of up to several minutes, which is in contrast to the rapid elevation of $[Ca^{2+}]_i$ previously observed in synaptosomes from rat brain (Meldahl & Fonnum, unpublished), and the prompt release of acetylcholine from nerve terminals in rat bronchial smooth muscle (Meldahl *et al.*, unpublished). However, such difference in toxin sensitivity between different preparations is not unreasonable. It has thus previously been reported that the toxin of *P. patelliferum* inhibits uptake of neurotransmitters more efficiently in synaptosomes than in synaptic vesicles from the rat brain (Meldahl *et al.,* 1994a). Moreover, the effect of the toxin of *P. patelliferum* is strongly dependent on the amount of target material (Meldahl *et al.*, 1995), which may complicate comparisons between the toxin sensitivity of different preparations.

Like *P. parvum*, *P. patelliferum* is likely to produce several toxic compounds, which may have different effects. Heterogenety of the algal extract was clearly demonstrated using the Ca^{2+}-channel blocker D-600. While this agent partly inhibited the increase in $[Ca^{2+}]_i$ induced by the algal extract, the toxin-induced lysis of the cells was not prevented or delayed. The toxic extract must thus have additional

effects to those revealed in the present study, either due to a compound with multiple effects, or to multiple compounds with different actions.

In conclusion, the toxic extract of *P. patelliferum* affects the Ca^{2+}-homeostasis of the GH_4C_1 cells by increasing the leak through voltage sensitive Ca^{2+}-channels, possibly by disclosing endogenous, inactivated Ca^{2+}-channels in addition to those being operational under control conditions. The resulting elevation of $[Ca^{2+}]_i$ facilitates hormone release from the cells.

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LEGENDS TO FIGURES

FIG. 1. Voltage dependent Ca^{2+}-currents in whole-cell voltage clamped GH_4C_1-cells. Upper recordings show representative inward Ca^{2+}-currents during depolarization to -7 mV from a holding potential of -67 mV in a control cell (a) and a cell exposed to toxic extract of *Prymnesium patelliferum* (70 000 cells ml^{-1}) (b). The current has both a transient and a sustained component. Lower diagrams show the current-voltage relation for the T-type (filled symbols, measured as the peak inward current) and L-type (open symbols, measured after 250 ms depolarization) currents. The recordings are from 67 cells in normal solution (c) and 57 cells in solution containing algal extract (50 000-100 000 cells ml^{-1}) (d). For each day of experimentation the current measurements were normalized as a percentage of the mean, transient current peak at -7 mV, and the values are presented as mean ± standard error. Triangles present recordings obtained with 100 μM D-600 in the control solution (n=10) and the toxin containing solution (n=9).

FIG . 2. Cytosolic Ca^{2+}-concentration in a single GH_4C_1 cell exposed to toxic extract of *Prymnesium patelliferum* (100 000 cells ml^{-1}). After recording for 10 min in normal extracellular solution, the medium was replaced with a corresponding solution containing the algal extract. The $[Ca^{2+}]_i$ was measured at two min intervals.

FIG. 3. Secretion of prolactin from GH_4C_1-cells during 15 min exposure to extract of *Prymnesium patelliferum* (70 000 cells ml^{-1}) in the absence of (hatched columns) and presence of (clear columns) 100 μM verapamil. Verapamil was applied 5 min before addition of the algal extract. The results are presented as % of the control, and represent the mean of two (verapamil containing solution) or three experiments performed in triplicates. Bars indicate standard deviation.

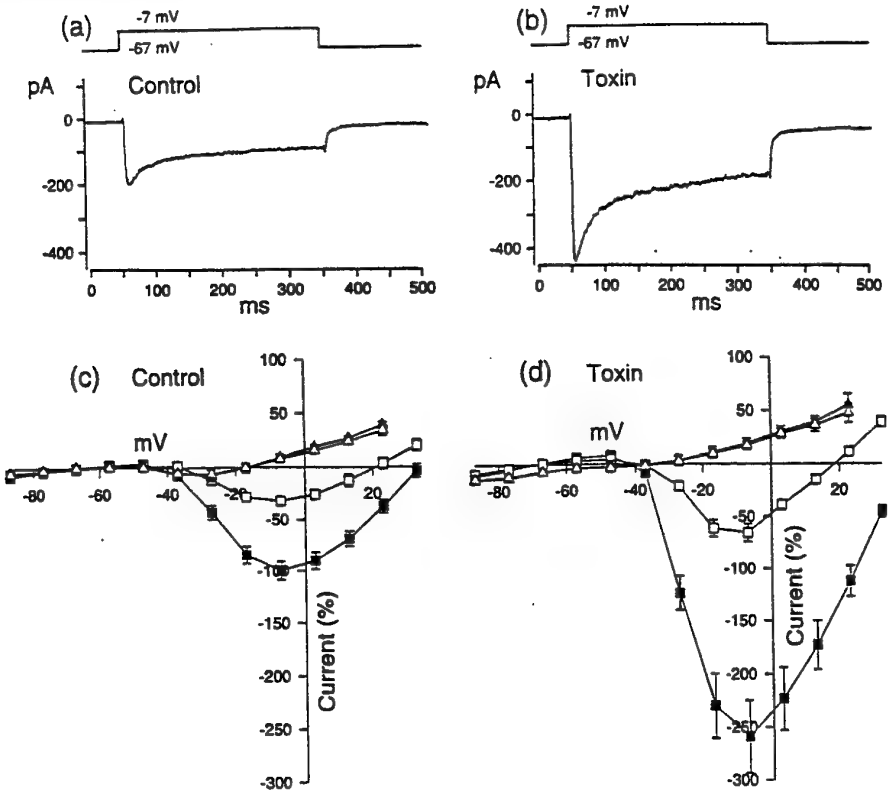

FIG. 1

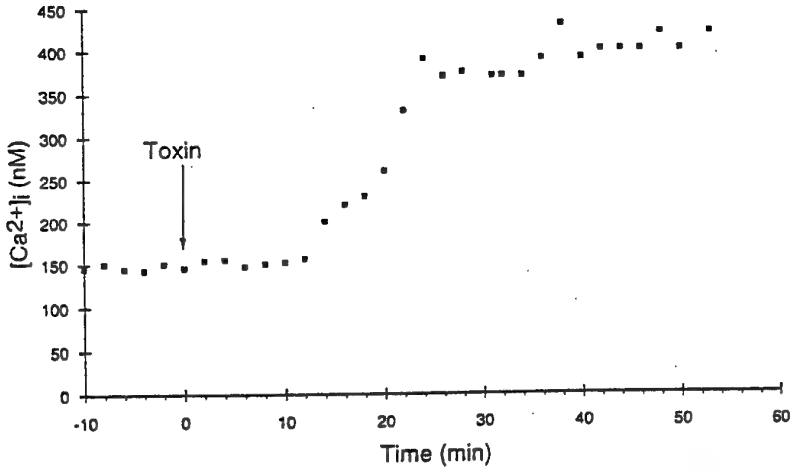

FIG. 2

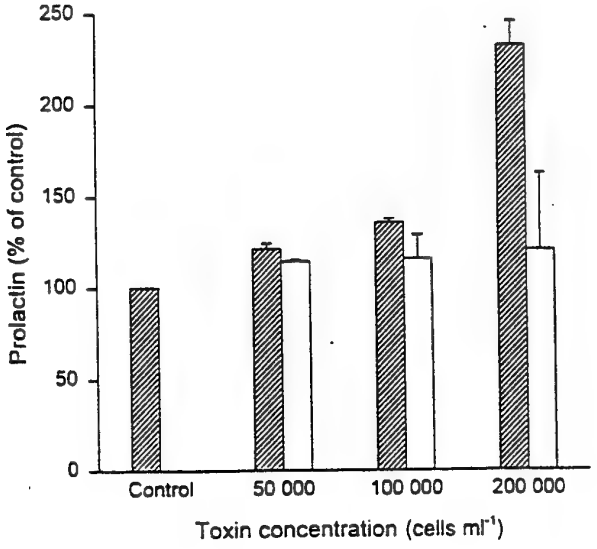

FIG. 3

PAPER V

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Paper V

European Journal of Pharmacology

Environmental Toxicology and Pharmacology Section

Extract of the marine alga *Prymnesium patelliferum* induces release of acetylcholine from cholinergic nerves

by

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Abstract

Previous studies on the toxic extract of the marine algal flagellate *Prymnesium patelliferum* have shown that it increased Ca^{2+} influx in synaptosomes and pituitary cells. It was therefore of interest to study the effect of the algal extract on neurotransmitter release. The algal extract enhanced the spontaneous as well as the K^+evoked (51 mM K^+) release of [^{3}H]acetylcholine and endogenous acetylcholine from autonomic cholinergic nerves of rat bronchial smooth muscle. The effects were concentration-dependent and reversible. The enhancement of the K^+ evoked release by the algal extract was partly dependent on extracellular Ca^{2+} and was significantly suppressed by the organic Ca^{2+} blockers ω-conotoxin GVIA (1 μM), diltiazem (100 μM), nifedipine (100 μM) and flunarizine (100 μM). The enhancement of the spontaneous release seemed Ca^{2+} independent and not sensitive to the Na^+ channel blocker tetrodotoxin. Sphingosine (20 μM), a protein kinase C inhibitor, strongly potentiated the enhancement of spontaneous release of [^{3}H]acetylcholine induced by the algal extract whereas another protein kinase C inhibitor, 1-(5-quinolinesulphonyl)-2-methylpiperazine (H-7) (20 μM), was without effect. A similar potentiation as seen with sphingosine, was observed with procaine (100 μM) and flunarizine (100 μM). The results indicate that the enhancement of the K^+ evoked release of [^{3}H]acetylcholine by the toxic extract of *P. patelliferum* was partly due to activation of voltage-dependent Ca^{2+} channels. The increase in the spontaneous release of [^{3}H]acetylcholine and endogenous acetylcholine induced by the algal extract alone may be due to an ionophore-like property of the algal extract. This effect of the algal extract may, moreover, be enhanced by compounds that facilitate the interaction of the algal toxin with the plasma membrane such as the lipophilic compounds flunarizine, procaine and sphingosine.

Keywords: Prymnesium patelliferum, *Prymnesium* toxin, Acetylcholine release, Ca^{2+} channel blockers

1. Introduction

The toxins produced by the marine algal flagellates *Prymnesium patelliferum* and *Prymnesium parvum* have in recent years been responsible for mass mortality of salmon and rainbow trout in aquaculture pens in Norwegian coastal waters (Eikrem and Throndsen, 1993). The two algal species are morphologically closely related and toxins with similar biological activities *in vitro* are present in lipid extracts of both algae (Meldahl et al., 1994a). The toxic extracts exert lysis of human red blood cells, and inhibit the uptake of the neurotransmitters glutamate and γ-aminobyturic acid (GABA) into synaptosomes and synaptic vesicles isolated from rat brain (Meldahl et al., 1994a). The toxin(s) of *P. patelliferum* have not been chemically characterized. In *P. parvum*, the quantitatively dominating hemolytic compound was suggested to be a mixture of two closely related digalactosyl monoglycerides with highly unsaturated fatty acid chains (Kozakai et al., 1982). Recently, however, Igarashi *et al.* (1993) isolated two closely related polyhydroxy-polyene-polyether compounds from *P. parvum* which showed strong hemolytic activity and induced influx of Ca^{2+} into rat glioma C6 cells. Increase in Ca^{2+} influx has been shown also for the toxic extract of *P. patelliferum,* both in rat brain synaptosomes and in cultured pituitary cells (Meldahl et al., 1994b).

Studies on neurotransmission performed by Parnas and co-workers using various muscle preparations showed a broad spectrum of effects of the toxic extract of *P. parvum* (Parnas et al., 1963; Bergmann *et al.*, 1964; Parnas and Abbott, 1965). Of particular interest was the dual effect of the *P. parvum* toxin on the isolated guinea-pig ileum smooth muscle; the toxin initially caused a slow contraction of the gut followed by a reduced response to acetylcholine which gradually returned after repeated washings (Bergmann et al., 1964). It was suggested that the algal extract caused a presynaptic efflux of acetylcholine. However, no direct evidence for this was provided in the study at that time.

The aim of the present study was to examine the mechanism of action of a toxic extract of *P. patelliferum* on the peripheral cholinergic nervous system. The bronchial smooth muscle of the rat was used as a model (Aas and Helle, 1982), since the tissue is highly enriched with cholinergic nerves and is a "pure" muscarinic preparation (Barnes, 1986). Depolarization of this

preparation by a high concentration of K^+ evokes Ca^{2+} dependent release of acetylcholine (Aas and Fonnum, 1986). We report here that the toxic extract of *P. patelliferum* enhanced both the Ca^{2+} dependent K^+ induced release of [^{3}H]acetylcholine and endogenous acetylcholine as well as the Ca^{2+} independent spontaneous release of [^{3}H]acetylcholine and endogenous acetylcholine. Using Ca^{2+} channel blockers and other agents previously reported to interact with the presynaptic neurotransmitter release process, we have studied the mechanism of action of the algal extract on the release of [^{3}H]acetylcholine from the bronchial cholinergic nerves.

2. Materials and methods

2.1. Animals

Male Wistar rats (200-300 g; from Møllegaard, Copenhagen, Denmark) were used throughout, and the rats were given a standard laboratory diet and water *ad libitum*. The animals were kept in standard laboratory cages, six in each, for 1-2 weeks before the start of the experiments. The sawdust bedding was replaced daily to ensure that the concentration of ammonia was kept at a low level. The light:dark cycle was 12 hours, relative humidity 45-55% and temperature 22-25°C. The rats had no sign of symptoms of respiratory tract infections. They were killed by decapitation, the bronchi were removed and transferred to a physiological buffer (see *2.4 Release of [^{3}H]acetylcholine*).

2.2 Chemicals

[^{3}H]-(methyl)-choline chloride (80.0 Ci $\times$ mmol^{-1}) was purchased from New England Nuclear (Boston Mass., USA). Acetylcholine chloride, diltiazem hydrochloride, flunarizine dihydrochloride, hemicholinium-3, procaine, sphingosine, tetrodotoxin, verapamil hydrochloride and 1-(5-quinolinesulphonyl)-2-methylpiperazine (H7) were purchased from Sigma Chemical Company (St. Louis, MO, USA). ω-Conotoxin GVIA was from Peninsula Laboratories Inc. (Belmont, CA, USA), and nifedipine from Research Biochemicals Inc. (Mass., USA). 1,2-bis(*o*-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxy methyl (BAPTA/AM) was from Molecular Probes Inc., (Oregon, USA). Soman (o-[1,2,2-trimethylpropyl]-methyl-phosphonofluoridate) (99% pure) was synthesized in this laboratory. All other chemicals were of analytical laboratory reagent grade. Due to light sensitivity, nifedipine was dissolved in ethanol immediately before use and protected from exposure to light.

2.3. Growth and extraction of algal cultures

The axenic strain of *Prymnesium patelliferum* (strain N) originated from a sample collected from a toxic algal bloom in Hylsfjorden, south-western Norway, 3 August 1989, and was grown as previously described (Meldahl et al., 1994a). The cell concentration was determined in an electronic particle counter (Coulter Electronics Ltd., model D). Crude toxin extracts were obtained by extraction as described by Ulitzur and Shilo (1970). In brief, one volume of the concentrated algal culture was mixed with 4 volumes of methanol-chloroform (1:2), and the lower chloroform fraction was evaporated to dryness in a rotary evaporator. The dry extracts were dissolved in methanol and stored in darkness at -20°C without loss of activity until testing.

2.4. Release of [^{3}H]acetylcholine

The release of newly synthesized [^{3}H]acetylcholine from cholinergic nerves of the smooth muscle of rat bronchi was determined by the method previously described by Aas and Fonnum (1986). The primary bronchi were opened ventrally and cut into small pieces (5 × 1 mg wet wt). The tissue was loaded with 1.1 μM [^{3}H]choline chloride (80 Ci × mmol^{-1}) at 25°C for 60 min with continuous shaking in normal buffer (see below). After incubation, the tissue was washed twice in normal buffer, and superfused for 60 min before collection of the samples. The superfusion chambers were made of two disposable pipette tips which were cut in half transversely and joined together. A flow rate of 200 μl × min^{-1} was generated by a peristaltic pump (Ole Dich Instrumentmakers Amp, Denmark, 6 channels). The normal superfusion buffer contained (in mM): NaCl, 140.0; KCl 5.1; $CaCl_2$, 2.0; $MgSO_4$, 1.0; Na_2HPO_4, 1.2; Tris-HCl, 15.0; glucose, 5.0; pH 7.4. The depolarization buffer contained 51 mM KCl and the concentration of NaCl was reduced accordingly to keep the ionic strength constant. In buffer with low Ca^{2+} concentration (0 mM) the Mg^{2+} concentration was raised to 10 mM 5-40 min before the second stimulation (S2). During the experiment the superfusion media also contained hemicholinium-3 (10 μM) to inhibit the high affinity uptake of choline (Yamamura and Snyder,

1973). The media were kept in a thermostatically controlled water bath at 25 °C and continuously gassed with 100% O_2. The release of [^{3}H]acetylcholine was induced by raising the K^+ concentration to 51 mM for 5 min. At this concentration of K^+ the release of [^{3}H]acetylcholine was 50% of maximum release obtained with 100 mM K^+ (Aas et al., 1987). Three 5 min periods of stimulation with high K^+concentration (S1, S2 and S3) were separated by 35-40 min superfusion with normal buffer. The effects of the algal extract or other agents were studied by treatment only during the second stimulation (S2) while the first (S1) and the third (S3) stimulation with high K^+ concentration were carried out as in the control experiment. Algal extract was present in the superfusion buffer during the 5 min stimulation (S2) either with high K^+ concentration (51 mM) or normal physiological buffer (5.1 mM K^+). The spontaneous release of [^{3}H]acetylcholine represents basal release of [^{3}H]acetylcholine from cholinergic nerves and choline metabolites as [^{3}H]phosphorylcholine. Treatment with nifedipine, diltiazem, flunarizine or tetrodotoxin started 5 min before and continued through the stimulation (S2). ω-Conotoxin GVIA, procaine, sphingosine and BAPTA/AM were present 30 min before and during S2. In the latter case Ca^{2+} was removed 10 min previous to and during the treatment with BAPTA/AM. Treatment with a low Ca^{2+} concentration (0 mM) started 40 min before S2. The collected fractions (1 ml) of the superfusion media were counted in 5 ml OPTI-FLUOR scintillation coctail. Homogenized tissue (1.5 ml) was counted for estimation of the total content of [^{3}H] in the tissue.

2.5. Release of endogenous acetylcholine

The release of endogenous acetylcholine from the cholinergic nerves in rat bronchi was determined by high performance liquid chromatography (HPLC) with electrochemical detection previously described by Stadler and Nesselhut (1986). The experiments with algal extract (625 000 cells x ml^{-1}) were performed as described for the determination of the release of [^{3}H]acetylcholine (*2.4 Release of [^{3}H]acetylcholine*) in the presence of high (51 mM) (5 min) and low (5.1 mM) K^+ concentration, but in the absence of hemicholinium. Prior to superfusion,

the tissue was incubated (15 min) in 1 μM soman to completely inhibit the cholinesterase activities (Aas et al., 1987). The superfusion buffer, 1 ml fractions, were freeze-dried (approximately 12 h) and resuspended in 200 μl distilled water. Samples (20 μl) of the supernatants were analyzed directly. Runs with standards of acetylcholine were carried out several times daily.

2.6. Data analysis

The presence of hemicholinium-3 during the experiment prevented the presynaptic reuptake of [^{3}H]choline. The total increase in [^{3}H] (i.e. [^{3}H]acetylcholine and [^{3}H]choline) efflux from the bronchial tissue pieces was thus taken as a relative measure of released [^{3}H]acetylcholine (Alberts et al., 1982; Wessler et al., 1990; Aas et al., 1987). The spontaneous release of [^{3}H]acetylcholine was subtracted from the evoked release, and the release of [^{3}H]acetylcholine in each of the three subsequent stimulations (S1, S2 and S3) was calculated as percent of that released in the first stimulation with high K^+ concentration (S1) in each experiment. The potassium evoked release of endogenous acetylcholine in the presence of algal extract was calculated as pmol x mg w.w.$^{-1}$ and and compared to control release without algal extract.

The mean and standard error of the mean (S.E.M.) was calculated for all data. Statistical analyses were done with Student's *t*-test (two-tailed).

3. Results

The release of [^{3}H]acetylcholine and endogenous acetylcholine from the superfused bronchial tissue pieces was induced by raising the K^+ concentration to 51 mM for 5 min. Figure 1A shows the relative amount of [^{3}H]acetylcholine released in three sequential stimulations in the presence of normal physiological concentration of Ca^{2+} (2.0 mM). Addition of the toxic extract of *P. patelliferum* at a concentration corresponding to 625 000 cells $\times$ ml^{-1} during the 5 min second stimulation (S2) with high K^+ concentration, enhanced the release of [^{3}H]acetylcholine (Fig. 1B) compared to high K^+ concentration alone (Fig. 1A). Also under normal physiological conditions (K^+ concentration=5.1 mM), the presence of this concentration of algal extract for 5 min (S2) induced a significant (12 fold) increase in the release of [^{3}H]acetylcholine compared to basal release (Fig. 1C and 1D). It should be noted that the release of [^{3}H]acetylcholine obtained by the combination of high K^+ concentration and algal extract was higher than the sum of the separate releases obtained by algal extract under basal conditions and by high K^+ concentration alone. In all experiments the effect of the toxic extract was reversible, as the subsequent stimulation (S3) with high K^+ concentration, after a 35 min washing period in the absence of toxic extract, was not different from S3 in the control experiment.

The effect of the algal extract when present in combination with high K^+ concentration, or in the normal physiological buffer with low K^+ concentration, was concentration-dependent (Table 1, A-H). The concentration of algal extract corresponding to 625 000 cells $\times$ ml^{-1} was used in all the subsequent experiments in order to investigate the mechanism of action of the toxic extract of *P. patelliferum*. Methanol, the extract vehicle (final concentration 0.3 %) showed no detectable effect on the release of [^{3}H]acetylcholine from the cholinergic nerves (data not shown).

To verify that the effects of the toxin(s) were on the release of acetylcholine from cholinergic nerves, we also measured the effects of the toxin(s) on the K^+ evoked (51 mM) release and its effect on the spontaneous release of endogenous acetylcholine. The K^+ evoked and the spontaneous release of endogenous acetylcholine were significantly enhanced in the

presence of algal extract (625 000 cells x ml^{-1}) (Table 2, B and D). The effect on the release of acetylcholine was reversible. In the presence of algal extract and high K^+ concentration the acetylcholine release was enhanced by approximately 85 % (Table 2, B), while the algal extract alone enhanced the spontaneous acetylcholine release by approximately 866 % (Table 2, D) compared to the basal release.

In agreement with previous studies (Aas and Fonnum, 1986) treatment with low Ca^{2+} concentration (0 mM) and high Mg^{2+} concentration (10 mM) for 5 min before and during S2 resulted in a marked reduction in the K^+ induced release of [^{3}H]acetylcholine (Table 3, A). Furthermore, the enhancement of the K^+ induced release in the presence of the algal extract was also significantly reduced by lowering the extracellular Ca^{2+} concentration (Table 3, B), indicating a possible Ca^{2+} dependence for the enhancement of [^{3}H]acetylcholine release by the algal extract in combination with sustained depolarization with high K^+ concentration. In contrast, the release of [^{3}H]acetylcholine induced by the algal extract under normal physiological conditions with low K^+ concentration was not altered by lowering the extracellular Ca^{2+} concentration (Table 3, C). To investigate the role of intracellular free Ca^{2+} in the effect of the toxin of *P. patelliferum*, the muscle preparation was treated with the membrane-permeant Ca^{2+} chelator BAPTA/AM (30 μM) in Ca^{2+} free buffer 20 min before and during the second stimulation (S2) in order to bind the cytosolic free calcium. This treatment did, however, not reduce the release of [^{3}H]acetylcholine induced by the extract of *P. patelliferum*, neither in the presence nor in the absence of high K^+ concentration (Table 3, D-F).

The involvement of voltage-dependent Ca^{2+} channels during K^+ evoked release of [^{3}H]acetylcholine was studied using blockers of different types of Ca^{2+} channels. Exposure to ω-conotoxin GVIA, nifedipine, diltiazem or flunarizine in rather high concentrations gave small but significant inhibition of the release of [^{3}H]acetylcholine evoked by sustained depolarization with high K^+ concentration (Table 4, A, D, G, J). The release of [^{3}H]acetylcholine induced by the algal extract in combination with high K^+ concentration was attenuated by all the Ca^{2+} channel blockers to about 50% of that released with high K^+ concentration and algal extract in the absence of blocker (Table 4, B, E, H, K). Under normal

physiological conditions, however, none of the Ca^{2+} channel antagonists were able to attenuate the enhancement of the spontaneous release by the algal extract (Table 4, C, F, I, L, M). There was a rather unexpected effect of flunarizine when present together with the algal extract under normal physiological conditions. While having no significant effect alone (data not shown) flunarizine at 100 μM and 10 μM caused a ten-fold and four-fold increase, respectively, in the release [^{3}H]acetylcholine induced by the algal extract (Table 4, L, M).

To investigate a possible role of Na^+ in the release of [^{3}H]acetylcholine induced by the algal extract, the bronchial preparation was treated with tetrodotoxin (3 μM) or procaine (100 μM), agents that block the voltage-dependent Na^+ influx by binding to different sites on the Na^+ channel (Pauwels et al., 1986). Neither of the compounds were able to inhibit the release of [^{3}H]acetylcholine evoked by the addition of algal extract to the resting muscle preparation (Table 5, B-C, E-F). Procaine, however, strongly potentiated the release induced by the algal extract (Table 5, E-F). We were unable to pursue the role of Na^+ in the effect induced by the algal extract by replacing the extracellular Na^+ concentration by an impermeant cation, because removal of extracellular Na^+ is itself toxic to the bronchial tissue preparation.

It was of interest to investigate whether extract of *P. patelliferum* could interact directly with factors involved in the neurotransmitter release process downstream to the voltage activated influx of Na^+ and Ca^{2+}. We studied the effect of inhibitors of the Ca^{2+}/phospholipid-dependent protein kinase, protein kinase C, which functions as an important regulatory element of a nearly ubiquitous signal transduction system in mammalian cells (Robinson, 1991). The bronchial preparation was treated with either of two inhibitors of protein kinase C, sphingosine (20 μM) (Hannun et al., 1986) and H-7 (20 μM) (Hidaka et al., 1984). Sphingosine caused a marked potentiation of the release of [^{3}H]acetylcholine evoked by the algal extract under resting conditions (Table 5, I), whereas H-7 was without effect (Table 5, L). Neither of the agents affected the release of [^{3}H]acetylcholine in the absence of algal extract under resting or depolarized conditions.

4. Discussion

The purpose of the present study was to investigate the effects of the toxic extract of *P. patelliferum* on the presynaptic release of acetylcholine and whether the observed effects involved alteration of the influx of Ca^{2+}. We have used a well defined preparation of the rat bronchial smooth muscle tissue which upon depolarization with high K^+ releases [^{3}H]acetylcholine in a Ca^{2+} dependent manner (Aas and Fonnum, 1986). The results of these studies show that the toxic extract of *P. patelliferum* enhanced both the spontaneous and the K^+ evoked release of [^{3}H]acetylcholine as well as endogenous acetylcholine from the cholinergic nerves in the smooth muscle tissue. These results indicate that the effects of the algal toxic extract are apparently mainly due to a specific effect on the release of acetylcholine and not a result of a hemolytic activity followed by liberation of phosphorylcholine or of other radioactiovely labelled metabolites.

Low extracellular Ca^{2+} concentration (0 mM) as well as treatment with the Ca^{2+} channel blockers, ω-conotoxin GVIA (1 μM), nifedipine (100 μM), diltiazem (100 μM) and flunarizine (100 μM) markedly suppressed the enhancement of the K^+ evoked transmitter release elicited by the algal extract, indicating a role of Ca^{2+} in the action of the extract and a possible effect on voltage-dependent Ca^{2+} channels. On the other hand, under resting conditions (low K^+ concentration), the release of [^{3}H]acetylcholine induced by the algal extract was not inhibited by low extracellular Ca^{2+} concentration, by chelating intracellular Ca^{2+} with BAPTA, or by any of the Ca^{2+} channel blockers, indicating a Ca^{2+} independent mechanism of action. From these results it seemed as there were at least two factors or mechanisms involved in the enhancement of [^{3}H]acetylcholine release caused by the extract of *P. patelliferum*: one dependent of extracellular Ca^{2+} shown only in combination with sustained depolarization with high K^+ concentration, and one (or more) independent of Ca^{2+}.

The algal extract seemed to act synergistically rather than additively on the release evoked by high K^+ concentration since the relative amount of [^{3}H]acetylcholine released by the addition of algal extract in combination with high K^+ concentration (342 ± 61 %) was higher than the sum of that released by algal extract (79 ± 15 %) and by high K^+ concentration (83 ±

2 %) in separate experiments. A possible explanation to this synergistic effect might be that the algal extract revealed voltage-dependent Ca^{2+} channels that were closed during depolarization with 51 mM K^+ alone. This is consistent with the finding that the blockers of voltage-dependent Ca^{2+} channels (ω-conotoxin GVIA, nifedipine, diltiazem and flunarizine) were relatively more effective in attenuating the release of [^{3}H]acetylcholine induced by the combination of high K^+ concentration and the algal extract, than that induced by high K^+ concentration in the absence of the algal extract. Furthermore, the results correspond with the results of previous electrophysiological studies on cultured pituitary cells using the whole-cell voltage-clamp technique in which increase in Ca^{2+} influx through endogenous Ca^{2+} channels caused by the extract of *P. patelliferum* was highest at depolarized potentials (Meldahl et al., 1994b).

To our surprise some of the compounds (flunarizine, procaine and sphingosine), which were examined because of their potential in reducing neurotransmitter release during depolarization, potentiated the release of [^{3}H]acetylcholine induced by algal extract. The mechanism for this potentiation is not clear. This effect of flunarizine was unexpected since under depolarized conditions with high K^+ concentration, flunarizine did not elicit such potentiation of the release in the presence of algal extract. Although used as a blocker of the transient T-type Ca^{2+} channels (Takahashi and Akaike, 1991; Wang et al., 1990), flunarizine is considered non-specific as calcium channel antagonist. The precise mode of action of flunarizine remains speculative. It binds to phospholipid bilayers and shows dissimilar interaction with different phospholipid classes (Thomas and Verkleij, 1990). Being amphiphilic and able to penetrate the phospholipid bilayer (Thomas and Seelig, 1993), flunarizine could combine with the toxin(s) of *P. patelliferum*, which is probably also of amphiphilic character (Kosakai et al., 1987; Igarashi et al., 1993), thereby mediating its interaction with the plasma membrane, resulting in increased effect of the algal toxin. Procaine and sphingosine are also of amphiphilic character, and are likely to interact similarly with the algal extract. Moreover, both flunarizine and procaine have affinity for the Na^+-channel (Pauwels et al., 1986; Pauwels et al., 1989; Kiskin et al., 1993) and by binding to the algal toxin these compounds could direct the toxin to the Na^+ channel resulting in a facilitation of the action of the algal toxin with the

presynaptic membrane. An increase in Na^+ influx would be consistent with the increased permeability of synaptosomes to Na^+ in the presence of the algal extract (Meldahl and Fonnum, unpublished). The role of increased Na^+ influx in the release of [^{3}H]acetylcholine induced by the algal extract remains to be studied in more detail using a preparation which is resistant to modulations of extracellular Na^+ concentration.

In conclusion, we suggest that the ability of the toxic extract of *P. patelliferum* to enhance the release of [^{3}H]acetylcholine/acetylcholine from cholinergic nerves in smooth muscle was partly due to an activation of voltage-dependent Ca^{2+} channels in addition to the channels activated by sustained depolarization with 51 mM K^+, and partly to an effect independent of Ca^{2+}. The algal extract may contain a compound with ionophoretic properties that may depolarize the nerve due to abolishment of ion gradients leading to increased efflux of [^{3}H]acetylcholine. Moreover, the effect of the algal extract may be enhanced by compounds that facilitate the interaction of the algal toxin(s) with the plasma membrane such as the lipophilic compounds flunarizine, procaine and sphingosine.

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Legend to Figure 1

Fig. 1. The effect of the toxic extract of *P. patelliferum* on the K^+ evoked (51 mM) and the spontaneous release of [^{3}H]acetylcholine from cholinergic nerves in pieces of rat bronchial smooth muscle. Release of [^{3}H]acetylcholine was induced three times (S1, S2, S3) [A and B] or two times (S1, S3) [C and D] by raising the K^+ concentration from 5.1 mM to 51 mM for 5 min. The algal extract at a concentration corresponding to 625 000 cells $\times$ ml^{-1} was present in the superfusion medium during the 5 min second stimulation (S2) with a high K^+ concentration (51 mM) (B) or under normal physiological conditions (5.1 mM K^+) (D). The release of [^{3}H]acetylcholine in S1 was defined as 100% (105 071 $\pm$ 10 732 dpm $\times$ g^{-1} wet wt $\times$ min^{-1}, n=15) and the release of [^{3}H]acetylcholine in S2 and S3, is presented as percent of the release in S1 (mean $\pm$ S.E.M. of three to nine experiments). The spontaneous [^{3}H]acetylcholine release during a stimulation period of 5 min (S1) was 227 555 $\pm$ 13 958 dpm $\times$ g^{-1} wet wt $\times$ min^{-1}, n=15. For significance for difference between groups, see Table 1.

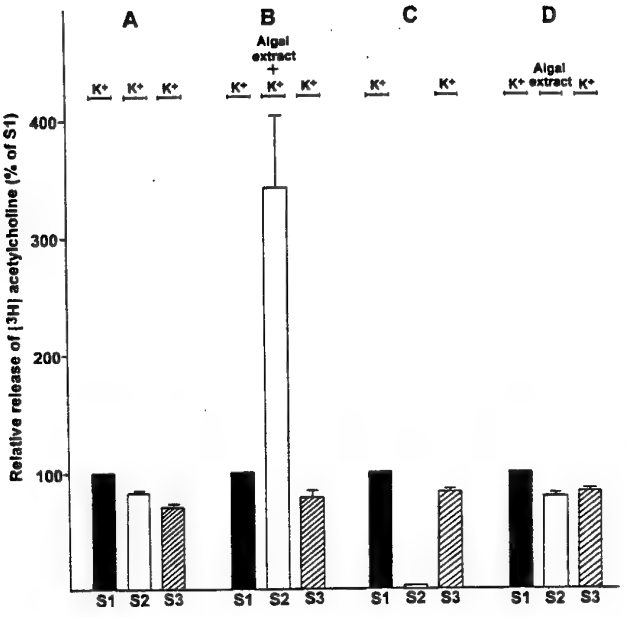
A
B
C
D
Algal extract
+
K+
Algal extract
Relative release of [3H] acetylcholine (% of S1)
400
300
200
100
S1 S2 S3 S1 S2 S3 S1 S2 S3 S1 S2 S3

Table 1

The concentration-dependent effect of the toxic extract of *P. patelliferum* on the K^+ induced and the spontaneous release of [^{3}H]acetylcholine from cholinergic nerves in pieces of rat bronchi.

	Concentration of algal extract present in S2 (cells × ml^{-1})	Stimulation in S2	Release of [^{3}H]acetylcholine					
			S1 (%)	S2 (% of S1)		S3 (% of S1)		n
A.	Control	K^+	100	83 ± 2		71 ± 2		29
B.	125 000	K^+	100	75 ± 6	d (A)	58 ± 4	d (A)	3
C.	312 000	K^+	100	172 ± 12	a (A)	67 ± 1	d (A)	3
D.	625 000	K^+	100	342 ± 61	a (A)	79 ± 5	d (A)	9
E.	Control		100	6 ± 2		93 ± 7		3
F.	125 000		100	12 ± 1	d (E)	92 ± 3	d (E)	3
G.	312 000		100	35 ± 11	d (E)	81 ± 7	d (E)	3
H.	625 000		100	79 ± 15	a (E)	83 ± 2	d (E)	6

Release of [^{3}H]acetylcholine was induced three times (S1, S2, S3) (A - D) or two times (S1, S3) (E-H) by raising the K^+ concentration from 5.1 mM to 51 mM for 5 min. The algal extract was present in the superfusion buffer only during the 5 min period of the second stimulation (S2). Each stimulation was separated by washing with normal buffer for 35 min. The release of [^{3}H]acetylcholine in S1 was defined as 100% and the release of [^{3}H]acetylcholine in S2 and S3, is presented as percent of the release in S1 (mean ± S.E.M. of n experiments). Significance for difference from S2 and S3 in control experiments with no toxic extract present (experiment (A) or (E)), was calculated for each S2-value and S3-value using Students *t*-test (two-tailed). a: $P < 0.01$, d: $P > 0.05$ (not significant)

Table 2

The effect of the toxic extract of *P. patelliferum* (625 000 cells x ml^{-1}) on the K^+ induced (51 mM) and spontaneous release of endogenous acetylcholine from cholinergic nerves in pieces of rat bronchi.

	Concentration of algal extract present in S2 (cells x ml^{-1})	Stimulation in S2	Release of endogenous acetylcholine (pmol x mg w.w.$^{-1}$)			
			S1	S2	S3	n
A	Control	K^+	1.02±0.07	0.69±0.12	0.59±0.20	4
B	625 000	K^+	1.17±0.17 d (A)	1.27±0.16 a (A)	0.86±0.11 d (A)	8
C	Control		0.01±0.01	0.03±0.02	0.06±0.02	6
D	625 000		-	0.29±0.03 a (C)	-	6

Release of endogenous acetylcholine was induced three times (S1,S2,S3) (A,B) or two times (S1,S3) (C) by raising the K^+ concentration from 5.1 mM to 51 mM for 5 min. The algal extract was present in the superfusion buffer only during the 5 min period of the second stimulation (S2)(B,D). Each stimulation was separated by washing with buffer for 35 min. The release of acetylcholine is measured as pmol x mg w.w.$^{-1}$ (mean ± S.E.M. of n experiments). Significance for difference from S1, S2 and S3 (A and B) (C and D) in control experiments with no toxic extract present was calculated for each S2-value and S3-value using Student *t*-test (two tailed). a: $P < 0.01$, d: $P > 0.05$ (not significant)

Table 3

The effect of the toxic extract of *P. patelliferum* (625 000 cells × ml^{-1}) on the K^+ induced (51 mM) and spontaneous release of [^{3}H]acetylcholine from cholinergic nerves in pieces of rat bronchi after a reduction in the extracellular concentration of Ca^{2+} in the superfusion buffer and after chelating the intracellular free calcium with BAPTA.

Treatment in S2	Stimulation in S2	Release of [^{3}H]acetylcholine					n
		S1 (%)	S2 (% of S1)		S3 (% of S1)		
A. Ca^{2+} (0 mM)	K^+	100	13 ± 1	a (A)	72 ± 7	d (A)	3
B. Ca^{2+} (0 mM) + Algal extract	K^+	100	150 ± 11	b (D)	102 ± 5	a (D)	8
C. Ca^{2+} (0 mM) + Algal extract		100	69 ± 9	d (H)	78 ± 4	d (H)	3
D. Ca^{2+} (0 mM) + BAPTA/AM (30 µM)	K^+	100	23 ± 4	d (A)	69 ± 3	d (A)	3
E. Ca^{2+} (0 mM) + BAPTA/AM (30 µM)+Algal extract	K^+	100	137 ± 10	d (B)	103 ± 6	d (B)	6
F. Ca^{2+} (0 mM) + BAPTA/AM (30 µM)+Algal extract		100	146 ± 16	a (C)	96 ± 6	d (C)	6

Release of [^{3}H]acetylcholine was induced three times (S1, S2, S3) or two times (S1, S3) as indicated by raising the K^+ concentration from 5.1 mM to 51 mM for 5 min. The algal extract was present in the superfusion buffer only during the 5 min period of the second stimulation (S2). Each stimulation was separated by washing with normal buffer for 40 min. The release of [^{3}H]acetylcholine in S1 was defined as 100% and the release of [^{3}H]acetylcholine in S2 and S3, is presented as percent of the release in S1 (mean ± S.E.M. of n experiments). Significance for difference from S2 and S3 in control experiments indicated by a letter in parenthesis (values for experiment (A), (D) and (H) are shown in Table 1 (experiments A-C) and values for experiments (A), (B) and (C) are shown in Table 3 (experiments D-F) was calulated for each S2-value and S3-value using Students *t*-test (two-tailed). a: $P < 0.01$, b: $P < 0.02$, d: $P > 0.05$ (not significant)

Table 4

The effect of the toxic extract of *P. patelliferum* (625 000 cells × ml^{-1}) on the enhancement of K^+ induced and spontaneous release of [^{3}H]actetyl-choline from cholinergic nerves in pieces of rat bronchial smooth muscle after treatment with Ca^{2+} channel blockers.

	Treatment in S2	Stimulation in S2	Release of [^{3}H]acetylcholine					n
			S1 (%)	S2 (% of S1)		S3 (% of S1)		
A.	ω-Conotoxin (1 μM)	K^+	100	67 ± 3	a (A)	63 ± 3	d (A)	6
B.	ω-Conotoxin (1 μM) + Algal extract	K^+	100	151 ± 14	b (D)	64 ± 5	c (D)	5
C.	ω-Conotoxin (1 μM) + Algal extract		100	68 ± 21	d (H)	48 ± 3	a (H)	3
D.	Nifedipine (100 μM)	K^+	100	66 ± 2	a (A)	79 ± 4	d (A)	9
E.	Nifedipine (100 μM) + Algal extract	K^+	100	168 ± 23	c (D)	72 ± 5	d (D)	3
F.	Nifedipine (100 μM) + Algal extract		100	61 ± 9	d (H)	79 ± 7	d (H)	3
G.	Diltiazem (100 μM)	K^+	100	44 ± 2	a (A)	70 ± 2	d (A)	9
H.	Diltiazem (100 μM) + Algal extract	K^+	100	165 ± 25	c (D)	68 ± 7	d (D)	6
I.	Diltiazem (100 μM) + Algal extract		100	131 ± 22	d (H)	61 ± 2	a (H)	6
J.	Flunarizine (100 μM)	K^+	100	74 ± 2	c (A)	52 ± 2	d (A)	6
K.	Flunarizine (100 μM) + Algal extract	K^+	100	194 ± 27	c (D)	37 ± 3	b (D)	6
L.	Flunarizine (100 μM) + Algal extract		100	755 ± 96	a (H)	55 ± 3	a (H)	6
M.	Flunarizine (10 μM) + Algal extract		100	318 ± 91	d (H)	63 ± 4	a (H)	6

Release of [^{3}H]acetylcholine was induced three times (S1, S2, S3) or two times (S1, S3) as indicated by raising the K^+ concentration from 5.1 mM to 51 mM for 5 min. The algal extract was present in the superfusion buffer only during the 5 min period of the second stimulation (S2) in the presence or absence of high K^+ concentration. Each stimulation was separated by washing with normal buffer for 35 min. The release of [^{3}H]acetylcholine in S1 was defined as 100% and the release of [^{3}H]acetylcholine in S2 and S3, is presented as percent of the release in S1 (mean ± S.E.M. of n experiments). Significance for difference from S2 and S3 in control experiments shown in Table 1 (experiments (A), (D) or (H)) was calulated for each S2-value and S3-value using Students *t*-test (two-tailed). a: $P < 0.01$, b: $P < 0.02$, c: $P < 0.05$, d: $P > 0.05$ (not significant)

Table 5

The effect of the toxic extract of *P. patelliferum* (625 000 cells × ml^{-1}) on the enhancement of K^+ induced and spontaneous release of [^{3}H]actetylcholine from cholinergic nerves in pieces of rat bronchi after treatment with the Na^+ channel blockers tetrodotoxin (TTX) and procaine, and the protein kinase C inhibitors sphingosine and H-7.

Treatment during S2	Stimulation in S2	Release of [^{3}H]acetylcholine					n
		S1 (%)	S2 (% of S1)		S3 (% of S1)		
A. TTX (3 μM)	K^+	100	82 ± 4	d (A)	82 ± 6	d (A)	6
B. TTX (3 μM) + Algal extract	K^+	100	245 ± 22	d (D)	76 ± 4	d (D)	3
C. TTX (3 μM) + Algal extract		100	86 ± 10	d (H)	84 ± 3	d (H)	3
D. Procaine (100 μM)		100	6 ± 7	d (E)	79 ± 16	d (E)	6
E. Procaine (100 μM) + Algal extract		100	386 ± 68	a (H)	86 ± 8	d (H)	9
F. Procaine (50 μM) + Algal extract		100	223 ± 67	d (H)	88 ± 4	d (H)	3
G. Sphingosine (20 μM)		100	10 ± 3	d (E)	87 ± 6	d (E)	6
H. Sphingosine (20 μM)	K^+	100	96 ± 7	d (A)	80 ± 5	d (A)	3
I. Sphingosine (20 μM) + Algal extract		100	894 ± 136	a (H)	85 ± 6	d (H)	6
J. H-7 (20 μM)		100	4 ± 1	d (E)	84 ± 3	d (E)	3
K. H-7 (20 μM)	K^+	100	80 ± 4	d (A)	64 ± 4	d (A)	3
L. H-7 (20 μM) + Algal extract		100	57 ± 12	d (H)	80 ± 3	d (H)	3

Release of [^{3}H]acetylcholine was induced three times (S1, S2, S3) or two times (S1, S3) as indicated by raising the K^+ concentration from 5.1 mM to 51 mM for 5 min. The algal extract was present in the superfusion buffer only during the 5 min period of the second stimulation (S2) in the presence or absence of high K^+ concentration. Each stimulation was separated by washing with normal buffer for 35 min. The release of [^{3}H]acetylcholine in S1 was defined as 100% and the release of [^{3}H]acetylcholine in S2 and S3, is presented as percent of the release in S1 (mean ± S.E.M. of n experiments). Significance for difference from S2 and S3 in control experiments shown in Table 1 (experiments (A), (D), (E) and (H)) was calulated for each S2-value and S3-value using students *t*-test (two-tailed). a: $P < 0.01$, d: $P > 0.05$ (not significant)

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